JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

Volume 22
Number 2

February 1959

DEPOSITED BY THE
UNITED STATES OF AMERICA

pCO_2 Inhibition of Normal and Malignant Growth [1,2]

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Summary

Evidence is presented for the theory that normal adult cells limit their own growth by producing inhibitory levels of free CO_2 consequent to their own respiration. It is suggested that embryonic and malignant cells achieve the ability to grow continuously by using an adenosine triphosphate (ATP) generating system—glycolysis—that does not result in the production of CO_2. Embryonic cells lose this auxiliary system as they mature into adult cells, but cancer cells develop glycolytic potential as they become malignant. Evidence from several sources suggests that free CO_2 is a physiologically produced, nontoxic, reversible inhibitor of cell growth. Experiments by Root suggest that this effect may be achieved by limiting the supply of ATP consequent to the self-inhibition of respiration by its end product, CO_2. If normal respiring cells are prevented from growing by a deficiency in their supply of metabolic energy, then embryonic and cancer cells may escape this physiological control by producing additional amounts of ATP through glycolytic mechanisms. To paraphrase Armin Braun's description of the crown-gall tumor cell, this suggests that, as a result of the transition from a normal cell into a fully malignant cancer cell, a specific growth-substance synthesizing system—glycolysis—becomes progressively activated. This leads to the production by the cancer cell of greater than regulatory amounts of this growth fcator—ATP. The continued production of this substance by the cancer cell could account for the unregulated proliferation of such a cell.—**J. Nat. Cancer Inst. 22: 207–217, 1959.**

Homeostasis has been defined by Walter Cannon as the tendency to stability found in such normal body states as blood sugar, temperature, and alveolar CO_2 (*1*). One aspect of this over-all concept may be termed cytostasis, *i.e.*, the tendency to stability of both the total number and different types of cells that constitute an adult organism. Cytostasis, in other words, refers to the self-inhibitory equilibrium existing between metazoan cells, which prevents *net* growth except after injury. Unlike a population of protozoa, metazoan cells do not grow indefinitely but reach a stable condition of cytostasis in the adult organism.

[1] Received for publication September 17, 1958.

[2] One of a series of speculative papers received in response to the open invitation which appeared in previous issues.

Little is known of the forces responsible for cytostasis. What biochemical factors initiate growth after injury and yet control it so that growth ceases once the injury has been repaired? Why is the growth of cancer cells not controlled by the same forces that maintain cytostasis in normal tissues?

One possible mechanism was suggested by Armin Braun *(2)*. He found that plant cells would not grow in a basic medium of mineral salts and sucrose unless 7 specific growth substances were added: glutamine, asparagine, inositol, cytidylic and guanylic acids, 6-furfurylaminopurine, and naphthaleneacetic acid. In contrast, crown-gall tumor cells would grow in the absence of all these growth factors.

This result suggests that differentiated plant tissues have lost the power to synthesize their own growth substances. Their rate of growth is apparently controlled by the regulated amount of these growth substances carried to them from distant parts of the intact plant. As Braun states, "It thus appears that, as a result of the transition from a normal cell to a fully altered, rapidly growing crown-gall tumor cell, a series of quite distinct, but well-defined growth-substance-synthesizing systems becomes progressively activated. This leads to the production by the affected cell of greater than regulatory amounts of these growth-promoting substances. The continued production in greater than regulatory amounts of these substances by the tumor cell could and most probably does account for the continued unregulated proliferation of such a cell." (*2*).

Is such a mechanism responsible for cytostasis and malignancy in animal tissues? The answer would appear to be negative on first consideration, because feeding excess vitamins, amino acids, or nucleic acids to animals does not alter their adult form. Growth hormones appear to be special rather than general regulators of tissue growth because they control the growth of only certain tissues, and some animals, such as hydra, demonstrate cytostasis even though they contain neither endocrine cells nor a circulatory system. No animal cancer cell, moreover, has ever been found that can manufacture its own growth hormone.

If lack of externally supplied growth substances is not responsible, perhaps cytostasis results from one or more self-produced inhibitors. Meryl Rose has postulated a whole hierarchy of organ-specific inhibitors (*3*). In a recent review of inhibitor theories (*4*), Rose suggested that ". . . malignant tumors may fail in self-limitation because excessive cell death prevents them from achieving limiting concentrations."

The present paper presents evidence for a new theory, namely, that cytostasis results from an insufficient supply of phosphate bond energy—adenosine triphosphate (ATP)—consequent to the *in vivo* inhibition of respiration by its end product, CO_2.

pCO_2

Writing over a generation ago, Haywood and Root (*5*) stated, "In two respects carbon dioxide claims a place of peculiar interest for the physiologist. First, as a metabolic product, it is present wherever there are active

cells. Secondly, as many workers have observed, it has the effect of very promptly depressing certain cell activities." Despite this and similar statements in the literature, tissue pCO_2 has been curiously neglected for many years. Perhaps this neglect stems from the fact that ". . . pCO_2 is the most troublesome of all biological factors to measure" (*6*), and any variable that is hard to measure is likely to be neglected.

Loomis has surveyed the chemistry and biology of pCO_2 (*7*, *8*), and has emphasized that three new methods of measuring this important feedback variable have recently been devised [3] (*8*, *9*), so that, today, pCO_2 can be measured and controlled experimentally with considerable precision.

pCO_2 refers to the pressure of CO_2 gas dissolved within a tissue, just as pO_2 refers to the pressure of dissolved oxygen gas. It differs from total CO_2 in that it is not concerned with such nongaseous products as H_2CO_3, HCO_3 and CO_3 except as they buffer the existing concentration of free, unhydrated CO_2 molecules. CO_2 gas is physiologically unique because, being un-ionized, it is more soluble in fat than in water (*10*) and hence is able to pass through nuclear and cell membranes faster than any other known substance (*11*, *12*). Photosynthesizing plants, for example, are known to absorb free CO_2 molecules rather than the almost impermeable bicarbonate ion (*13*). Since all respiring tissues generate CO_2, they automatically create gradient fields of pCO_2 that permeate tissues almost as if they were acellular. Any particular cell within a respiring tissue, therefore, is under the influence of the over-all pCO_2-gradient of that tissue.

Some idea of the physiological importance of pCO_2 is given by the care with which the respiratory center of the brain regulates pCO_2 rather than oxygen. Powers (*14*) has pointed out that lung-breathing animals have the dual advantage of living in air that contains a fixed percentage of CO_2 (0.03%) and of possessing closed alveolar spaces within which alveolar CO_2 can be closely regulated at 5.3 percent CO_2. Fish, he pointed out, have gills that are essentially open systems; they can control their arterial pCO_2 only by *a*) regulating their level of muscular activity or by *b*) migrating to water of higher or lower pCO_2. He suggested that this explains why active brook trout inhabit aerated brooks where the pCO_2 approximates 0.03 percent atmosphere, while lethargic lake trout inhabit the depths of partially stagnant lakes where the pCO_2 may be ten times higher.

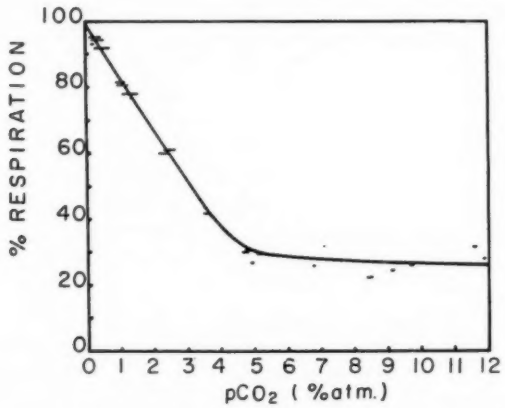
Given the importance of tissue pCO_2 as a feedback variable, what is its level in human tissues? Venous and capillary blood have a pCO_2 of about 46 mm. Hg or 6 percent atmosphere, so that human tissues contain gradients of pCO_2 that begin at about 6 percent atmosphere and rise progressively the farther a cell is from an open capillary (to perhaps 15–20% atm.). The height of this gradient depends *a*) on the respiratory rate of the tissue and *b*) on the number of open capillaries within it. Since tissue respiration and vascularity often increase at the same time,

[3] Direct pCO_2 measurements may be made with the new CO_2-pH Duometer manufactured by Advanced Instruments, Inc., 45 Kenneth Street, Newton Highlands 61, Mass.

it may be that tissue pCO_2 is homeostatically regulated in the same manner as alveolar pCO_2.

pCO_2 Inhibition of Respiration

Root (*15*) has shown that pCO_2 specifically inhibits respiration in fertilized *Arbacia* eggs (text-fig. 1).



TEXT-FIGURE 1.—pCO_2 inhibition of respiration in fertilized *Arbacia* eggs. Data from figure 3 of (*15*).

Since similar experiments with tissues from higher animals do not seem to have been carried out,[4] this curve may be used to obtain some indication of how pCO_2 affects cellular respiration in general. The danger in such an extrapolation may not be as great as it first appears, for many years of work have shown that the respiratory and glycolytic enzymes of cells are surprisingly alike throughout the plant and animal kingdom. Yeast and pigeon-breast muscle, for example, are both classical sources of respiratory enzymes.

Text-figure 1 presents several interesting points. First, a pCO_2 of 6 percent atmosphere is seen to inhibit respiration by over 70 percent. Since cell survival requires only about one fifth the energy needed for maximum cell growth (*16*), this suggests that cytostasis results from the 6 to 15? percent atmosphere pCO_2 present in adult mammalian tissues, for 70 to 80 percent inhibition of respiration would generate only enough ATP to maintain tissues without providing them with enough ATP for growth.

Second, any lowering of tissue pCO_2 would strongly increase the supply of ATP available to a cell and hence tend to initiate growth. A surface abrasion of the skin, for example, would allow CO_2 to be lost directly to

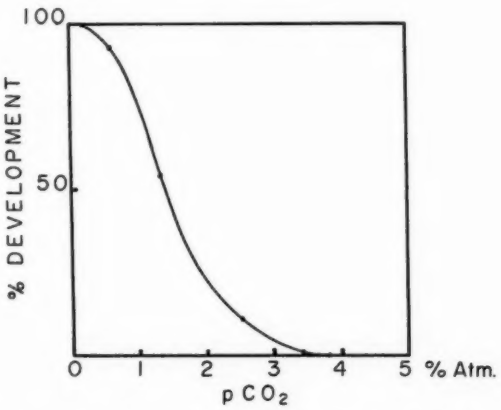
[4] Added in proof: In subsequent experiments, Root found that increased levels of pCO_2 inhibited the respiration of both nerve and muscle. *See* Root, W. S.: The influence of carbon dioxide upon the respiration of nerve. J. Cell. & Comp. Physiol. 1: 239–252, 1932; and Root, W. S.: The influence of carbon dioxide upon the respiration of muscle. Physiol. Zool. 6: 137–149, 1933.

the air and hence would tend to lower the pCO_2 of the newly exposed cells. Alternatively, any increase in vascularity to an area would tend to lower its pCO_2 and thus stimulate growth. Removing cells to the environment of a tissue culture would also tend to initiate growth, if the pCO_2 of the tissue-culture air space were lower than it had been *in situ*. Finally, text-figure 1 is interesting in that the curve flattens out above about 6 percent atmosphere, and thus allows maintenance levels of ATP to be generated even under very high pressures of CO_2.

Maintenance of Cytostasis by Regulated Levels of pCO_2

Direct evidence that CO_2 is a physiologically produced, nontoxic, and reversible inhibitor[5] of cell growth is available from work on lower animals. Goldin (*17*), for example, found that appropriate levels of pCO_2 can cause complete inhibition of growth in regenerating *Tubularia* tissues. Smith and Clowes (*18*) found that pCO_2 progressively inhibits cell division in both sea urchin (*Arbacia*) and starfish (*Asterias*) eggs (text-fig. 2).

Haywood and Root (*5*) confirmed these findings, including the important point that complete inhibition of growth was obtainable by sufficient elevation of the pCO^2. Salisbury and VanDemark (*19*) showed that a pCO_2 of 100 percent atmosphere reversibly inhibited both the glycolytic activity and motility of mammalian sperm, the reversible nature of such inhibition being shown by finding that sperm stored in a glass ampoule



TEXT-FIGURE 2.—pCO_2 inhibition of cell division in fertilized *Asterias* eggs. Data from figure 3 in (*18*).

[5]Evidence from several sources (*7, 8*) indicates that low levels of pCO_2 stimulate cell differentiation, even though high levels are inhibitory to growth and respiration. This finding may explain the morphological inferiority of glycolytic to respiratory ATP as seen, for example, in the dedifferentiation of cancer cells and in the anaerobic *versus* aerobic culture of yeast. Warburg (*16*) believes that structural differences explain this phenomenon, *i.e.*, respiratory ATP is generated within mitochondria, while glycolytic ATP is generated within the fluid protoplasm. Since ATP from any source probably equilibrates rapidly throughout the cell, it seems more likely that the morphogenetic superiority of respiratory ATP stems from the fact that it alone is accompanied by the generation of CO_2.

under CO_2 was fully fertile after the rigors of a week at room temperature (*20*). Normally, of course, sperm remains fertile only when stored under refrigerated conditions.

Taken together, these different experiments suggest that adult tissues may achieve cytostasis by limiting their supply of ATP through pCO_2 inhibition of their respiratory mechanism. It is unfortunate that similar studies have not been made on mammalian tissue cultures, a fact that is due perhaps to the mistaken notion that pCO_2 affects only pH (*21*).

Glycolytic Freedom From pCO_2 Inhibition

Phosphate bond energy can be produced in two alternative ways: 1) by respiration which yields CO_2 as its end product and 2) by glycolysis which does not yield CO_2 but lactic acid instead. If each of these enzyme systems tends to be specifically inhibited by its own end products, then glycolysis should be more resistant to pCO_2 inhibition than respiration. This prediction has been confirmed in recent experiments by Salisbury and VanDemark (*19*) who showed that sperm maintains 95 percent of its glycolytic activity at a pCO_2 of 5 percent atmosphere. Cells with well-developed respiratory *and* glycolytic machinery, therefore, should be able to obtain maintenance levels of ATP from their pCO_2-controlled respiration and, in addition, should be able to obtain additional amounts of ATP for growth by glycolysis. If cytostasis, therefore, results from an insufficient supply of ATP in self-inhibited respiring cells, uninhibited growth should occur in cells containing the auxiliary power supply known as glycolysis. The interesting fact is that, except for a few tissues,[6] normal adult tissues have low rates of glycolysis (*i.e.*, 1–8), while both embryonic and cancer tissues have high rates (*i.e.*, 16–70) (*22, 23*). Both embryonic and cancer cells, of course, are continuously growing tissues.

Glycolysis and Cancer

Warburg has long maintained that cancer cells originate in two phases: an irreversible injury to their respiratory apparatus, followed by a slowly developed increase in their glycolytic potential (*16*). Weinhouse (*24*) and others have argued that often there is no detectable reduction of respiratory activity in tumors. All workers are agreed, however, that malignant and embryonic cells characteristically show rates of glycolysis far above normal adult tissues. Warburg (*16*), in fact, found glycolytic rates as high as 70 in ascites tumor cells and remarked that with ". . . such high values we come very close to the fermenting values of wildly proliferating *Torula* yeasts." Weinhouse believes that ". . . high glycolysis occurs *despite* quantitatively and qualitatively normal occurrence of (respiratory) carbon and electron transport, . . ." concluding, "I believe it would be more accurate to state that anaerobic glycolysis is so high in tumours that a

[6] Special factors may operate in such tissues. For example, some may have lost the power to grow under any circumstances (brain and retina), others may translate their growth into special channels (myeloid and placental tissues), while still others may have lost so much of their respiratory capacity that their net rate of ATP synthesis is not sufficient for growth, despite their high glycolytic rate.

normal respiration and a normal Pasteur effect are incapable of eliminating it."

Much of the confusion surrounding the metabolism of tumors derives from the fact that the respiratory and the glycolytic *potential* of cells are operationally measured by the usual techniques, rather than their actual rates as they occur in the intact animal. Thus, respiration is usually measured in a Warburg vessel containing NaOH in a center well so that the pCO_2 approximates 0.0 percent atmosphere rather than the 6 to 15? percent atmosphere actually present in living tissues. All such studies, therefore, measure maximum, uninhibited respiratory rates rather than the 70 percent inhibited rates existing in normal tissues. Glycolysis similarly is measured under unphysiologically anaerobic conditions so that it, too, is measured under maximal, uninhibited conditions. Actual rates of glycolysis *in vivo* would be far lower, consequent to end-product inhibition by accumulated lactic acid and by the inhibition of glycolysis by respiration known as the Pasteur effect. Even the latter is a feedback entity, for respiration itself *in vivo* is not maximal but self-inhibited in nature, so that its effect on glycolysis is less than if it were running at full speed. Warburg today believes that *in vitro* measurements of aerobic glycolysis are meaningless ". . . because aerobic fermentation is too labile and too dependent on external conditions." (*16*).

What is needed today, therefore, is a study of the respiratory and glycolytic rates of normal and malignant tissues under as physiological conditions of pCO_2, pH, pO_2, and pNH_3 (*8*) as possible. Respiratory studies of this nature may be made either with the indirect method of Warburg (*in 25*) or with chemical rather than manometric methods of measuring oxygen uptake (*26*).

Respiration and Glycolysis in Normal and Malignant Tissues

Burk (*27*) has compared the respiratory and glycolytic potential of four homologous rat tissues: *i.e.*, normal, regenerating, embryonic, and cancerous (table 1).

TABLE 1.—*Comparison of respiration and glycolysis in homologous rat hepatic tissues* (24)

Tissue	Respiration	Glycolysis
	Q_{O_2}	$\overset{N}{Q_A 2}$
Normal	6.0	1.0
Regenerating	6.4	1.8
Embryonic	6.0	8.0
Hepatoma*	6.4	12.1

*Induced by feeding 4-dimethylaminoazobenzene.

Summarizing his results, Greenstein remarked, "What is indeed striking is the similar metabolic behavior of resting and regenerating liver, and the dissimilar behavior of the hepatoma. It is evident that, in the case of regenerating liver, it is possible to have rapid growth in the absence of

appreciable glycolysis. This is, however, controlled growth. In the case of the hepatoma, there is presented an ostensibly uncontrolled growth accompanied by extensive glycolysis. The four homologous rat hepatic tissues thus fall, metabolically, into two main categories, resting and regenerating liver on the one side and hepatoma and fetal liver on the other." (*23*). The conclusion seems to be that cells achieve cytostasis if they are powered by respiration alone, while they avoid cytostasis and grow continuously if they are powered by both respiratory *and* glycolytic mechanisms. Since normal cells obtain 99 percent of their ATP from respiratory sources (*16*), such cells achieve cytostasis by damping their furnaces with a blanket of CO_2. Embryonic and malignant cells, on the other hand, possess an auxiliary motor that is not inhibited by CO_2 and so is available to supply them with the extra energy needed for growth.

Why Does Carcinogenesis Occur in Normal Tissue Culture?

Biesele (*28*) has written that the enclosed environment of a tissue culture provides ". . . just the conditions needed to transform the metabolism of normal epithelial cells to that of malignant cells." Cancer, in other words, tends to arise spontaneously in normal cells grown for prolonged periods of time in tissue culture. Goldblatt and Cameron (*29*) suggested that this malignant change may be due to the intermittent anoxia that accompanies serially transplanted tissue cultures. Since incubating tissue cultures for 7 days at a time automatically exposes them to increasing conditions of anaerobiosis, long-continued cultures are repeatedly exposed to increasing levels of pCO_2 as well as to decreasing levels of pO_2. The operative variable in the malignant change therefore may actually be high pCO_2 rather than anoxia.

Earle *et al.* (*30*) have observed that the size of the inoculum in a culture has no effect on its final amount of growth. Growth is limited in such cultures by several conditions, including the size of the physical container and the accumulation of metabolites. Since the only way in which the size of container could affect the process is by limiting the diffusion of gases, it may be that self-limitation of growth results from the gradual accumulation of inhibitory levels of pCO_2.

If respiratory self-inhibition controls the growth of normal cells in tissue culture, what would happen if a cell should progressively mutate so that it possessed additional powers of glycolysis? Because any increase in the supply of ATP would increase the rate of growth of such a cell, one can predict that such respiring and glycolyzing cells would gradually dominate the culture, a process akin to natural selection operating to increase steadily their glycolytic potential with the continued passage of time.

Since small pieces of an old culture are used to start new ones, fortuitous selection of a mutated area could serve to speed this process by a mechanism akin to genetic drift. Tissue-culture routine, therefore, contains almost all the elements needed for rapid selection of mutant varieties.

For example, mutations themselves are fostered during the repeated bursts of growth that follow successive transplantations, for mutations chiefly occur during periods of active mitosis. Like a chronically irritated sore or mammary gland, bursts of growth alternate with periods of increasing cytostasis, the net result being a rapid increase in glycolytic potential and eventual malignancy.

Burk (*in 16*) has actually observed this process in two sublines, derived from the same identical normal cell that developed high and low malignancy at the same time that they developed high and low glycolytic potential. Strain 1742, for example, today produces cancer in 97 percent of mice injected and has a glycolytic potential of 70. Strain 2049, on the other hand, is apparently still precancerous, for it produces cancer in only 1 percent of mice injected and has a glycolytic rate of 25.

Whether glycolytic potential is gained by a process akin to somatic mutation (*31*) or by the "infection" of the strain with an extrinsic "virus" is immaterial to the argument, just as long as the altered cell is capable of transmitting its increased glycolytic potential to its descendents. In this connection, Burk (*32*) found that the Rous sarcoma 1 virus, which can induce malignancy within 7 days (*33*), confers both malignancy and increased glycolytic potential to the cells on which it acts.

Cancer as a Normal Biological Consequence of Cytostasis

It is a general biological truth that whenever a population is chronically suppressed by an inhibitor for prolonged periods, then eventually some members of the population become immune to the effect of the inhibitor and take over biologically. Just as new rusts continually appear in wheat-producing areas, and DDT-resistant flies appear in areas treated for several years with DDT, so perhaps the cancer cell represents the successful evolutionary response of cells grown under conditions of cytostasis where high levels of pCO_2 prevent their growth by starving them of metabolic energy. Just as streptomycin-resistant bacteria appear in cultures chronically suppressed with streptomycin, so cancer may represent ". . . a process as inevitable as evolutionary progress and of the same general quality." (*31*). It should be remarked in passing that the suppressive agent in observed instances of this phenomenon seems to act solely by selecting the favored mutant rather than by causing the mutation to occur in the first place.

Burnet has emphasized that the logarithmic relationship existing between cancer and increasing age bears all the earmarks of a series of progressive somatic mutations. "From the age of 20 or 30, it appears that we are subject to a random process becoming progressively more evident with age and with inexorable regularity . . . . The only way in which an observable effect can result is when mutants with a proliferative advantage can emerge. Every significant somatic mutation, whether affecting a normal cell or one that has already mutated, must necessarily be along the road to malignancy. The age incidence of cancer is precisely what would be expected from such a situation. In fact I

fancy that the log-log relationship between cancer incidence and age could be foreseen from the beginning as an inevitable consequence of the evolution of multicellular animals." (*31*).

In conclusion, if the biochemical uniqueness of the cancer cell resides in its dual mechanisms of ATP production, then any treatment that inhibits glycolysis, but not respiration, should be in the right direction. Total inhibition of glycolysis, of course, would have to be accompanied by the administration of lactic acid or some other source of respiratory substrate such as glutamate or acetate. Even then, glycolysis may be too fundamental a mechanism to inhibit safely, so that palliation rather than cure should be expected from all such methods. Actual cure of a cancer cell may be defined as its conversion into a normal cell, not by inhibiting its extra titer of glycolytic enzymes, but by preventing the synthesis of these enzymes in the first place. Since the synthesis of a high titer of glycolytic enzymes takes place in embryonic cells but not in adult ones, some normal process occurs *in vivo* that effectively inhibits the synthesis of these enzymes during maturation. If the mechanism of normal maturation can be elucidated, it may be possible to apply this knowledge to the problem of converting a malignant cell into a normal one.

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Grafted-Marrow-Rejection Mortality Contrasted to Homologous Disease in Irradiated Mice Receiving Homologous Bone Marrow [1,2]

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Summary

Bone marrow from the BALB/c and CBA inbred strains of mice and the (BALB/c × CBA)F_1 hybrid strain was injected isologously and in various homologous combinations into CBA and (BALB/c × CBA)F_1 hybrid mice irradiated with 330 r, 550 r, or 770 r (LD0, 12, and 100 respectively. At the sublethal dose of 330 r, no mortality resulted from any combination of marrow donor and recipient. At the higher doses, two types of mortality were observed in mice receiving bone marrow; (*a*) "early mortality" (5 to 21 days) after the ordinarily high sublethal dose of 550 r and (*b*) "delayed mortality" (21 to 60 days) following good, early protection against the otherwise lethal dose of 770 r. Neither type of mortality occurred after administration of isologous marrow, but both types occurred after administration of marrow from an unrelated homologous strain. When F_1 marrow was transferred into the parental strain (in which circumstance only the recipient strain is capable of immunologic reaction against the donor, but not *vice versa*) only "early mortality" was observed. When parental marrow was transferred into the F_1 hybrid (in which circumstance only the donor strain is capable of immunologic reaction against the recipient, but not *vice versa*) only "delayed mortality" was observed. From these observations and other considerations it is concluded that the early mortality, resulting from administration of homologous or heterologous marrow into mice irradiated in the high sublethal or midlethal dose range, is the result of the recovered ability of the host to reject the temporarily grafted marrow, whereas the delayed mortality, following good, early protection against otherwise lethal doses of irradiation with homologous or heterologous marrow, usually results from an immunologic reaction of the grafted marrow derivatives against the tolerant host. The former type of mortality was designated "grafted-marrow-rejection mortality"; the latter, "homologous disease" or "heterologous disease."—**J. Nat. Cancer Inst. 22: 219–228, 1959.**

Mammals of several species can be protected against otherwise lethal doses of whole-body X irradiation by postirradiation administration of

[1] Received for publication January 20, 1958.

[2] This work was supported by research grant C-3367 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[3] The author is indebted to Booker Morris, John Session, Ingrid Grups, and Johanna Angermann for technical assistance, and to Dr. R. Shalek for assistance in dosimetry.

X-ray facilities were provided by the M. D. Anderson Hospital and Tumor Institute.

bone marrow. Protection is associated with repopulation of the hematopoietic system by cells of the marrow donor, regardless of whether protection is achieved with isologous (same genotype), homologous (different genotype, same species), or heterologous (different species) bone marrow (*1*). Protection with homologous or heterologous bone marrow confers a tolerance to skin grafts from the marrow donor (or from the donor's parental strains if the marrow donor is an F_1 hybrid) as well as to the transplanted marrow (*2–5*). This tolerance represents a central failure of the immunologic response against tissue antigens from the marrow donor, since the tolerant state can be abolished by injection of normal lymphoid elements of the host genotype (*6*). The genetic relationship between marrow donor and irradiated recipient is of great importance with respect to the degree of initial protection, the duration of survival, and the induction of tolerance to skin grafts (*4, 7, 8*). Irradiated mice protected with isologous marrow show long-term survival and are not rendered tolerant of homologous skin grafts (*2–4*). Mice protected with homologous or heterologous marrow or infant spleen show irradiation recovery followed by a secondary loss of weight frequently resulting in death during the 2nd month post irradiation, without secondary hematopoietic depression (*4, 9*). Delayed mortality is more frequently encountered when the homologous marrow is from a donor of different histocompatibility-2 genotype (A into C57, DBA into A, and A into CBA) than when it is from a donor of the same *H-2* genotype (C3H into CBA) as the recipient (*4*). Protection with bone marrow from a coisogenic strain differing only at the *H-2* locus results in delayed mortality (*8*). This delayed mortality has been attributed by some investigators to a delayed immunologic reaction of the host against the grafted marrow elements (*10*) and, by others, to an immunologic reaction of grafted marrow elements against the tolerant host (*4, 7, 11, 12*).

During the course of experiments to determine the lowest dose of radiation, followed by homologous marrow, capable of conferring tolerance to skin grafts, a peculiar "zone-effect" was observed in the dose-mortality curve (*13*). In inbred strains of different *H-2* genotype, the parental strain irradiated and then treated with marrow from the F_1 hybrid was found to be tolerant of homologous skin from the other parental strain after 660 r or 770 r whole-body irradiation (30-day LD90 and LD100, respectively). Tolerance did not ensue when bone marrow was administered after 0, 110, or 330 r (LD0). After 550 r (LD12), skin grafting could not be performed since most of the marrow-treated mice died within 2 or 3 weeks after irradiation. The same marrow treatment that was necessary for protection against a lethal dose of radiation resulted in death when administered after a high sublethal dose! Death was preceded by a precipitous loss of body weight and by reduced circulating red and white blood-cell levels as compared to mice receiving marrow after the higher dose of 770 r.

The present investigation was undertaken to determine the combinations of marrow donor and recipient strains of mice in which this peculiar

sublethal-zone mortality might occur. Combinations of particular interest are those of parental strain marrow injected into F_1 hybrid mice and *vice versa*. The untreated F_1 hybrid mouse is genetically tolerant of tissue grafts from either inbred parental strain, but neither parental strain will accept tissue grafts from the F_1 hybrid. Since the sublethal-zone mortality was observed originally in an irradiated parental strain receiving marrow from an F_1 hybrid, it could conceivably have resulted from a recovered ability of the host to reject the temporarily transplanted marrow. It could, alternatively, result from something other than an immune reaction. If sublethal-zone mortality occurs when parental marrow is injected into the F_1 hybrid, it would indicate the latter possibility. If it failed to occur, it would indicate the former possibility.

Materials and Methods

CBA and BALB/c mice were used as parental strains for the F_1 hybrids. These two strains are not of the same *H-2* genotype (CBA = *H-2*^{k}; BALB/c = *H-2*^{d}). The CBA strain and the (BALB/c × CBA)F_1 hybrid were used as irradiated recipients. The doses of radiation and the combinations of donor and recipient strains are indicated in tables 1 and 2. These contain pooled data from several experiments. Wherever possible, an equal number of mice on each of the treatments used in a given experiment were housed together in one pen. Mice were randomized by age and sex in treatment groups. Irradiation conditions were: 200 kvp, 15 ma., 40 cm. target distance, 0.5 mm. Cu and 1.0 mm. Al filters, HVL = 1.3 mm. Cu, 85 to 90 r per minute dose rate. The 30-day LD50 for the CBA strain under these conditions is 600 r (*13*). Bone marrow was injected intravenously in a standard volume of 0.5 ml. per mouse. Where mixed marrow was administered, each mouse received 0.5 ml. of marrow suspension that was prepared by mixing equal parts of marrow suspension from each of the two strains. The mean of the nucleated cell counts of the suspensions used was 54,140 per mm.^{3} (standard deviation = 19,477). No variation in results was observed from one experiment to the next, which could be attributed to variation in the number of cells injected. Marrow was injected within 3 hours after irradiation.

Results and Discussion

The 30-day mortality for uninjected control mice at 330 r, 550 r, and 770 r was 0, 13, and 100 percent respectively for the CBA strain, and 0, 11, and 100 percent respectively for the (BALB/c × CBA)F_1 hybrids (tables 1 and 2).

At 330 r none of the combinations of marrow donor and recipient strains resulted in any early (5 to 21 day) or delayed (21 to 60 day) mortality. After 550 r, no early or delayed mortality, in either the CBA strain or the (BALB/c × CBA)F_1 hybrids, followed treatment with isologous marrow. In CBA mice receiving 550 r, progressive body-weight loss and early mortality followed the administration of marrow from either the F_1 hybrid or the unrelated BALB/c strain. However, in F_1

TABLE 1.—*Cumulative mortality in irradiated CBA mice*

X-ray dose (r)	Marrow donor strain	Number of mice	Percent mortality after indicated No. of days:							
			5	12	21	30	60	100	150	200
330	None	12	0	0	0	0	0	0	0	0
330	(BALB/c × CBA)F_1	36	0	0	0	0	0	0	0	3
330	CBA	12	0	0	0	0	0	0	0	0
330	BALB/c	12	0	0	0	0	0	0	0	0
550	None	23	0	9	13	13	13	13	13	13
550	(BALB/c × CBA)F_1	36	0	50	89	89	94	94	94	94
550	CBA	11	0	0	0	0	0	0	0	0
550	BALB/c	12	0	0	75	83	92	92	92	92
770	None	60	3	100	100	100	100	100	100	100
770	(BALB/c × CBA)F_1	72	3	6	7	10	19	21	22	25
770	CBA	60	3	7	7	10	13	15	20	20
770	BALB/c	36	0	6	14	28	58	64	64	67
770	BALB/c + CBA, mixed	24	0	0	0	0	4	4	4	4

hybrids receiving 550 r, no early mortality was obtained after the administration of marrow from either parental strain. Early mortality after otherwise sublethal irradiation was therefore seen only in those combinations in which the recipient strain could potentially reject tissue grafts from the donor strain, regardless of whether (BALB/c into CBA) or not (F_1 into CBA) the donor strain could react immunologically against the recipient strain. It was not seen in those combinations in which the recipient strain could not reject grafts from the donor strain (CBA into CBA, F_1 into F_1, CBA into F_1, and BALB/c into F_1), even though the donor strain could react immunologically against the recipient strain in the latter two combinations.

At 770 r, no comparable early mortality with secondary hematopoietic depression was seen in any combination. Delayed mortality was seen

TABLE 2.—*Cumulative mortality in irradiated (BALB/c × CBA)F_1 hybrid mice*

X-ray dose (r)	Marrow donor strain	Number of mice	Percent mortality after indicated No. of days:							
			5	12	21	30	60	100	150	200
330	None	10	0	0	0	0	0	0	0	10
330	(BALB/c × CBA)F_1	12	0	0	0	0	0	0	0	0
330	CBA	11	0	0	0	0	0	0	0	8
550	None	36	0	6	11	11	11	14	19	
550	(BALB/c × CBA)F_1	24	0	0	0	0	0	8	13	
550	CBA	24	0	0	8	8	17	21	29	
550	BALB/c	12	0	0	0	0	0	0	0	
770	None	48	6	79	98	100	100	100	100	
770	(BALB/c × CBA)F_1	24	0	8	8	8	13	13	13	
770	CBA	36	0	3	8	19	44	53	56	
770	BALB/c	35	0	6	6	8	8	11	14	
770	BALB/c + CBA, mixed	48	0	0	2	19	71	90	94	

with injections of BALB/c marrow into CBA, CBA marrow into F_1, and BALB/c plus CBA marrow into F_1, that is, only when the donor strain can react immunologically against the recipient strain, even though in the two latter combinations the recipient strain cannot react against the donor strain. The only combination of a parental marrow injected into an F_1 hybrid that failed to give delayed mortality after 770 r was BALB/c into (BALB/c × CBA). Delayed mortality in lethally irradiated F_1 hybrids protected with parental strain marrow was reported previously by Uphoff (*7*) and Trentin (*11*) for other combinations of parental strains. However, van Bekkum *et al.* (*14*) failed to obtain delayed mortality for still another combination of parental strains when parental marrow was injected into irradiated F_1 hybrids. It would appear, therefore, that when parental strain marrow is injected into F_1 hybrids after an otherwise lethal dose of radiation, delayed mortality may or may not occur, depending on the particular combination of strains used. In those F_1 hybrids that do not show delayed mortality after an injection of parental marrow, there may nevertheless be body weight loss, indicating that secondary disease may be present, but sublethal (*11*). Important factors in determining the severity of secondary disease are probably the degree of antigenic difference between the strains of mice used and the degree of lymphoid regeneration from the grafted marrow, since the homograft reaction is mediated by lymphoid cells. Data indicating lymphoid regeneration from marrow donor origin have been obtained by several laboratories (*15–19*), and Mitchison (*20*) demonstrated the production of antibodies by spleen cells transplanted from immunized donors into irradiated homologous mice.

At 770 r, delayed mortality is not shown when (BALB/c × CBA)F_1 marrow is injected into the CBA parent (table 1), even though the recipient strain can potentially reject tissue grafts from the donor strain, and even though this same combination gives high mortality of the early type after sublethal irradiation. A mixture of homologous and isologous marrow injected after 770 r (BALB/c plus CBA injected into CBA) prevents tolerance (determined by skin grafting) and averts delayed mortality, as previously reported (*11*).

The occurrence of the two types of mortality, with respect to the various types of strain combination used, and the immunologic potential of donor and recipient against each other are summarized in table 3. When neither host nor donor strain is capable of immunologic (histocompatibility) reaction against each other, then neither early mortality, after otherwise sublethal irradiation, nor delayed mortality, following early protection against otherwise lethal irradiation, is seen. When both host and donor strain are capable of reacting against each other, then both types of mortality occur. This indicates that both types of mortality are probably related to the immunologic incompatibility between donor and recipient. But foreign-strain homologous or heterologous combinations are incapable of indicating whether either type of mortality is related to incompatibility of host-against-graft or graft-against-host.

TABLE 3.—*Mortality following marrow injection in irradiated mice**

	Isologous	Homologous combinations		
	Neither host nor donor capable of histocompatibility reaction against each other	Both host and donor capable of reacting against each other	Host potentially capable of reacting against donor but not *vice versa*	Donor potentially capable of reacting against host but not *vice versa*
	Same genotype	Unrelated strains	F_1 marrow into parent	Parent marrow into F_1 hybrid
Five- to 21-day mortality after otherwise sublethal irradiation	−	+	+	−
Twenty-one- to 60-day mortality following early protection against otherwise lethal irradiation	−	+	−	+ or −

*Occurrence of the two types of mortality observed, with respect to strain combinations used, and the immunologic potential of donor and recipient against each other.

For this purpose, combinations of the F_1 marrow into the parental strain and parental marrow into F_1 hybrid are uniquely suitable since, in the former, only the host can react against the graft, while, in the latter, only the graft can react against the host. In these cases, "early mortality" is seen only with combinations of F_1 marrow into the parental strain, and not with parental marrow into the F_1 hybrid, whereas the reverse is true of "delayed mortality." It would appear, therefore, that "early mortality" after sublethal irradiation is related to a recovered ability of the host to reject the temporarily transplanted homologous marrow, whereas "delayed mortality" after otherwise lethal irradiation is the result of the transplanted homologous marrow (or its lymphoid derivatives) "rejecting" the tolerant host. This is in accord with the observed secondary hematopoietic depression in "early mortality" and the lack of it in "delayed mortality."

A sublethal or midlethal zone effect in mice receiving rat marrow was also observed by Congdon *et al.* (*21*) and by van Bekkum *et al.* (*14*), in mice receiving either rat marrow or foreign-strain mouse marrow but not isologous marrow. Congdon *et al.* nicely demonstrated a secondary degeneration of the transplanted bone marrow, and they were able to prevent mortality by administering isologous marrow 6 days after the rat bone marrow. This led them to the well-justified conclusion that such mortality results from a host-*versus*-graft reaction. However, they infer that because this type of mortality results from reaction of the host against the graft, the delayed mortality following protection against lethal irradiation with heterologous or homologous marrow is probably also caused by a host-*versus*-graft reaction (*21*, *10*). This is not in accord with the usual

lack of marrow depression in delayed mortality (*22*) or with the results obtained with F_1 marrow injected into the parental strain and with parental marrow injected into F_1 mice. Neither is it in accord with the long duration of tolerance to skin grafts of marrow-donor origin and the usually good condition of such skin homografts or heterografts at the time of delayed mortality (*4*, *5*). Nor is it in accord with the findings of Weyzen *et al.* (*23*) and of Grabar *et al.* (*12*) that the serum γ-globulin in mice protected against lethal irradiation with rat bone marrow is of rat origin.

An appropriate designation for the early mortality that results from rejection of the temporarily transplanted homologous or heterologous bone marrow in animals receiving whole-body irradiation in the high sublethal or midlethal dose range would appear to be "grafted-marrow-rejection mortality." The rejection of transplanted marrow resulted in the death of animals that would have had sufficient marrow recovery of their own to survive high sublethal irradiation if they had received no marrow injection at all. It must be postulated either that mortality results from toxic effects of the sudden rejection of large amounts of foreign tissue, or that the presence of the temporarily functional grafted-marrow prevents or delays the recovery of the host's own marrow. The finding of Congdon *et al.* (*21*) that death may be prevented by administration of isologous marrow 6 days after the administration of heterologous marrow favors the latter possibility.

The term "homologous disease" was used by Barnes *et al.* (*16*) to designate the delayed mortality observed in irradiated mice protected with homologous bone marrow or spleen, without reference to the etiology of such delayed mortality. The terms "homologous disease" and "heterologous disease" were proposed by Trentin (*11*) to designate the specific complications and mortality resulting from the successful transplantation of homologous or heterologous, as opposed to isologous or autologous, bone marrow or lymphoid tissue into a tolerant host, *whether irradiated or not*, and caused by a *graft*-versus-*host* reaction. Such mortality has been observed in unirradiated animals rendered tolerant by the transplantation of homologous spleen into the fetus or neonatal individual (*24*, *25*). It has also been produced by transplantation of lymphoid tissue from parental strain mice into their unirradiated but "genetically tolerant" F_1 hybrids (*26–28*) or into sublethally irradiated F_1 hybrids (*28*, *29*).

It is apparent that the dose of radiation is a very important determinant of the type of mortality resulting from the subsequent administration of homologous or heterologous bone marrow to mice. Doses of 330 r permit homograft rejection (*13*), but without mortality. In the high sublethal and midlethal dose ranges, a high mortality rate attends the rejection of grafted marrow. Above the MLD100, tolerance to the grafted marrow usually ensues in the systems studied, and the predominant cause of delayed mortality is homologous disease attributable to an immunologic reaction of the graft against the host. It is probable that between the latter two dose ranges both types of mortality (host-*versus*-graft, and

graft-*versus*-host) occur in the same population when heterologous or foreign-strain homologous marrow is used. Only by using the unique combinations of parental marrow into F_1, and *vice versa*, can the two types of mortality be clearly dissociated at all dose levels.

The possibility of wiping out one animal's hematopoietic system and substituting that of another by marrow transfusion suggests many intriguing possibilities, including the therapy of disseminated tumors of hematopoietic and other radiosensitive tissues with otherwise lethal doses of whole-body irradiation. Varying degrees of success have been achieved in this regard with transplanted animal tumors (*30–33*). Clinical application of such methods has already begun in several laboratories (*34*), but more obstacles exist in man than in the mouse, since there is no counterpart in man of an inbred mouse strain, and one is limited to identical twins for isologous marrow. Nor is there a counterpart in man of the F_1 hybrid homologous donor, which is unique in that it protects and induces tolerance to other tissues from the marrow donor without itself being immunologically reactive against the recipient parental strain. If successful transplantation of homologous bone marrow or lymphoid tissue is achieved in man, the severity of the reaction of graft-against-host cannot be predicted with certainty. It is hoped that the reaction will be minimal as in mice of the same *H-2* genotype. If not, it presents a serious obstacle unless typing for histocompatibility antigens in man is some day developed to the point that compatible tissue donors may be selected. Alternatively, it is possible that homologous disease may be minimized or averted by the use of fetal rather than adult donors. In this case, tolerance should theoretically be acquired by the graft as well as by the host. However, in this laboratory, experiments with fetal mouse liver as the source of homologous hematopoietic tissue for radiation protection have shown no significant advantage over the comparable adult bone marrow, with respect to homologous disease, in six strain combinations tested thus far. The possible use of marrow from agammaglobulinemic individuals to avert homologous disease has been suggested to the author by Dr. F. Gonzales.

The high mortality rate which, in mice, attends the rejection of temporarily grafted homologous bone marrow after a high sublethal or midlethal dose of irradiation, indicates another potential hazard in any clinical application of homologous bone-marrow transfusion after whole-body irradiation of humans.

The use of autologous marrow from the same individual would, of course, avoid the possibility of both grafted-marrow-rejection mortality and homologous disease. However, its applicability would be limited to those individuals whose bone marrow is both hematologically competent and free of malignant cells. Moreover, nothing could be gained by marrow removal and reinjection post irradiation that could not be achieved by shielding of areas of bone marrow *in situ* or by subtotal-body irradiation. Autologous marrow, however, does have potential utility in conjunction not with irradiation but with those cancer chemotherapeutic

agents whose use clinically is limited by a marrow-depressing effect. In this case it would be necessary to preserve the autologous marrow, during the period of chemotherapy, either by freezing (*35*) or by tissue culture (*36*, *37*).

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An Evaluation of Some Genetic Factors Influencing Irradiation Protection by Bone Marrow [1]

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Summary

A study of the genetic factors influencing the grafting of bone marrow in lethally irradiated mice revealed that a single gene difference at the *H*-2 locus provided sufficient basis for the severe reaction of the secondary phase of the irradiation syndrome. The *H-1* locus provided a variable reaction, while the *H*-3 locus produced no reaction. In the testing of 27 inbred-strain combinations, no combination was found in which good long-term survival was observed when the strains were of different *H*-2 phenotypes. Nine inbred-strain combinations in which the marrow donor and the recipient were of the same *H*-2 phenotype, either $H\text{-}2^b$ or $H\text{-}2^d$, were found to be compatible. Hybrids of the genotype $H\text{-}2^d/H\text{-}2^k$ were tested against strain A and gave further evidence that an immune response of the graft against the host was responsible for the secondary phase. A gene-dosage effect was proposed as a working hypothesis to explain the variability in response observed in F_1 hybrids treated with parental-strain marrow in contrast to the response found among different inbred-strain combinations. Evidence for a threshold effect in antigen-antibody response of F_1 hybrid mice treated with parental-strain marrow was discussed.—**J. Nat. Cancer Inst. 22: 229-241, 1959.**

Mice exposed to lethal, total-body X irradiation may survive the lethal effect of the treatment if they receive a postirradiation intravenous inoculation of intact bone marrow or splenic cells (*1*, *2*). It has been established that protection is a result of the repopulation of the host's hematopoietic system by the inoculated hematopoietic cells (*3–6*). In animals receiving homologous or heterologous marrow after lethal irradiation, there may be only a temporary recovery followed by increased mortality during the 2nd and 3rd months (*7*, *8*). There is considerable presumptive evidence indicating that this late mortality is the result of a homograft reaction of the marrow graft against the irradiated host (*9–15*).

[1] Received for publication September 23, 1958.

[2] The authors are grateful to Dr. G. D. Snell for making available the coisogenic lines used in these experiments; to Drs. M. K. Deringer and W. E. Heston for supplying the mice which made possible the extensive survey of different strain combinations; to Mr. Henry Meyer for his assistance in the irradiation of the animals used in this study; and to Drs. D. B. Amos, P. A. Gorer, and G. D. Snell for comments and suggestions during the preparation of this manuscript.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

In a study of genetic factors influencing the protection of the irradiated host by inoculation of homologous marrow, it was found that, in certain F_1 hybrids, the hybrid marrow afforded better protection than marrow of either parental strain (*13*, *14*). This effect was especially apparent in F_1 hybrids in which the parental strains had different genotypes at the *H-2* locus. This locus has been shown to be one of the important factors influencing the protection of lethally irradiated mice (*14*). Since there was some evidence that other histocompatibility loci may also influence this protection, an attempt was made to determine the importance of the other two identified histocompatibility loci, namely *H-1* and *H-3*, as well as to confirm the previously reported effect of the *H-2* locus by the use of a different pair of coisogenic lines. Additional data are presented on different inbred-strain combinations. Some of the salient information concerning genetic factors is evaluated in an attempt to reconcile the apparent incongruities between the data on irradiated F_1 hybrid mice (*13*, *16*) and those of different inbred-strain combinations.

Materials and Methods

Coisogenic lines.—Although there are numerous histocompatibility loci, only 3 have been genetically identified. The *H-2* locus is the strongest of the identified loci and has 17 known phenotypes (*17*). This locus not only regulates susceptibility and resistance to tissue transplantation but also determines a series of erythrocyte antigens. The *H-1* and *H-3* loci are weaker and less fully analyzed.

Snell (*18*, *19*) synthesized isogenic resistant lines of mice for his study on the relation of histocompatibility genes to susceptibility and resistance of tumor transplantation. One of the lines was a standard inbred strain; the other was synthesized by a series of genetic crosses and backcrosses that introduced onto the background of the inbred strain a small segment of chromosome carrying genes for graft resistance, which was introduced from the other parent of the initial cross. This small segment of chromosome introduced into the pure-strain background may also carry a marker gene as well as the histocompatibility gene. Theoretically it would be possible for two closely linked histocompatibility genes to be introduced on the chromosome segment, but there was no evidence for this in the coisogenic lines employed in these experiments (*20*).

The coisogenic pair, C3Hf and C3H.Kcc, differ at the *H-1* and the albino (*c*) loci. The coisogenic pair, ST and ST.T6, differ at the *H-2* locus, while C57BL/10 and B10.LP differ at the *H-3* and agouti (*A*) loci. The three sets of coisogenic lines used in the following experiments were obtained from Snell.

Inbred strains.—The inbred strains listed in tables 4 and 5 were from well-established, carefully inbred lines. They were either raised in this laboratory or were obtained from the colonies of Drs. M. K. Deringer and W. E. Heston. Their subline origin is as follows: C56BL/Ka, C57L/De, DBA/2Lw, BALB/cDe, CBA/Jax, AKR/Lw, A/De, C58/Lw, C57BL/10ScBs, C57BL/10(*H-2*^{d})/Bs, ST/Sn, ST.T6/Sn, C3Hf/He.

Experimental conditions.—At 3 to 4 months of age, mice were exposed to a 30-day LD98 of total-body X radiation, *i.e.*, 700 r or 800 r, depending on the strain treated. The LD98 was selected in an attempt to arrive at a standard biological effect of the irradiation on the different strains which vary in their radiosensitivity. Two X-ray tubes placed opposite each other were operated at 200 kvp and 15 ma.; filtration was 0.25 mm. Cu and 0.55 mm. Al with 54 cm. from each focal spot to the center of the mouse. The dose rate was 120 r per minute. All irradiated mice were randomized, and tail-vein inoculations of marrow from 4 long bone equivalents were completed within 3 hours of irradiation as previously described (*13*). Three groups were run concurrently: the irradiated control (noninoculated) group, the irradiated group receiving isologous marrow, and a group receiving coisologous marrow.

Results

As was shown previously (*13*), two distinct phases of the survival pattern following lethal total-body X irradiation and marrow inoculation are recognized: 1) the acute phase, which covers the first 14 days post irradiation—at which time the untreated control mice succumb, and 2) the secondary phase, which occurs only after the inoculation of certain homologous or heterologous marrow cells. Depending on the severity of the reaction during the secondary phase of the irradiation syndrome, there may be all degrees of survival from the reaction, *i.e.*, 100 percent survival to 100 percent lethality. The severity of the reaction varies with different graft-host combinations. Although it has been demonstrated that a tenfold increase in the number of homologous spleen cells will produce death earlier (*21*), there is no indication that the number of marrow cells inoculated has such an effect on the severity of the reaction during the secondary phase. A large inoculum was used to insure an adequate number of cells which would produce good protection from the acute irradiation damage in all strains treated.

Table 1 shows the survival pattern for mice of the coisogenic lines of the ST strain (Street) following exposure to 800 r total-body X radiation. The mice receiving isologous marrow showed good protection and good long-term survival. The mice receiving the coisologous marrow (ST.T6) showed an early secondary phase of an extreme type. There was severe diarrhea, extreme emaciation, dermatitis, and loss of weight. A typical

TABLE 1.—*Effect of isologous* (H-2^k) *or coisologous* (H-2^d) *marrow on survival of mice of strain ST following 800 r total-body irradiation**

Number of mice	Donor marrow	Donor phenotype	Percent survival at days:			
			14	30	60	90
34	ST	*H-2^k*	100	100	100	88
52	ST.T6	*H2^d*	94	44	23	11
45	None	None	2	0	0	0

*Data pooled from 5 experiments.

TABLE 2.—*Effect of isologous* (H-3^b) *or coisologous* (H-3^a) *marrow on survival of B10.LP mice following 700 r total-body irradiation**

Number of mice	Donor marrow	Donor phenotype	Percent survival at days:			
			14	30	60	90
96	B10.LP	*H-2*^{b}, *H-3*^{b}	73	70	67	67
115	C57BL/10	*H-2*^{b}, *H-3*^{a}	79	78	77	77
85	None	None	2	0	0	0

*Data pooled from 12 experiments.

comparison may be made between mice receiving isologous and coisologous marrow (fig. 1). Although the groups are small, they clearly demonstrate the influence of a gene difference at the *H-2* locus in producing an early, severe, and lethal reaction during the secondary phase of the irradiation syndrome.

Since in previous experiments (*14*) the long-term survival was relatively poor for irradiated C57BL/10 mice receiving isologous marrow, the B10.LP line was used as the irradiated host. Table 2 shows the survival pattern for these coisogenic lines which differ at the *H-3* locus. Although the initial protection through the acute phase is not particularly good, most animals which survived 14 days survived over 90 days, with no discernible reaction.

The coisogenic lines, C3Hf and C3H.K, differ at the *H-1* locus. The survival pattern of C3Hf mice treated with C3H.K marrow (table 3) is different from that of the other pair of coisogenic lines mentioned previously. Although the over-all survival is good and some animals show no gross evidence of a reaction, others show a "typical" reaction that is usually mild and to which few animals succumb.

Table 4 is a list of the inbred-strain combinations which were found to produce good long-term survival following a 30-day LD98 of X radiation and intravenous marrow inoculation. These recipient strains and the compatible donor strains share the same *H-2* phenotype. Since all combinations were not tested in both directions, there may be some variability which has not been detected, *i.e.*, a combination might be compatible in one direction but incompatible in the other direction. There are insufficient data available on strains of the *H-2*^{k} group to include these combinations in the tabulation at this time. Strains C3H and 101 are both

TABLE 3.—*Effect of isologous* (H-1^a) *or coisologous* (H-1^b) *marrow on survival of C3Hf mice following 700 r total-body irradiation**

Number of mice	Donor marrow	Donor phenotype	Percent survival at days:			
			14	30	60	90
64	C3Hf	*H-2*^{k}, *H-1*^{a}	86	78	78	75
100	C3H.K	*H-2*^{k}, *H-1*^{b}	93	92	75	73
68	None	None	1	1	1	0

*Data pooled from 10 experiments.

TABLE 4.—*Combination of inbred strains found to produce good long-term survival following lethal total-body irradiation and marrow inoculation*

Phenotype		Recipient strain	Donor strains (*percent survival at 90 days*)*
Recipient	Donor		
H-2^b	*H-2*^{b}	C57BL	C57L (100)
H-2^b	*H-2*^{b}	C57L	C57BL (100), C57BL/10 (100)
H-2^d	*H-2*^{d}	DBA/2	BALB/c (95)
H-2^d	*H-2*^{d}	BALB/c	DBA/2 (80)
H-2^d	*H-2*^{d}	C57BL/10 (*H-2*^{d})	DBA/2 (87), BALB/c (94), ST.T6 (80)
H-2^k	*H-2*^{k}	CBA/Jax	C3Hf (100)

*Percent calculated on 14-day survivors.

H-2^k (*22*), yet they have been found to be incompatible (G. E. Cosgrove, personal communication). Although some strain combinations within the *H-2*^{k} group are incompatible, there are compatible combinations within this group as is illustrated by the CBA-C3Hf combination (table 4).

Table 5 lists the inbred-strain combinations which have been found to be incompatible, *i.e.*, there is a reaction with a resulting, greatly reduced, long-term survival. These combinations all represent strains with different *H-2* phenotypes. Although inbred strains of the same *H-2* phenotype may be incompatible, no inbred-strain combination has been observed in which good long-term survival was successfully achieved when the strains were of *different H-2* phenotypes.

TABLE 5.—*Combinations of inbred strains shown to produce a reaction resulting in greatly reduced long-term survival after lethal total-body irradiation and marrow inoculation*

Phenotype		Recipient strain	Donor strains (*percent survival at 90 days*)*
Recipient	Donor		
H-2^a	*H-2*^{k}	A	AKR (40)
H-2^a	*H-2*^{b}	A	C57BL (0)
H-2^b	*H-2*^{d}	C57BL/10	C57BL/10 (*H-2*^{d}) (30)
H-2^b	*H-2*^{k}	C57BL	C3Hf (0), C58 (20)
H-2^d	*H-2*^{k}	BALB/c	C3Hf (38), C58 (0)
H-2^d	*H-2*^{k}	DBA/2	C3Hf (5), AKR (0)
H-2^d	*H-2*^{k}	ST	ST.T6 (11)
H-2^d	*H-2*^{a}	DBA/2	A (0)
H-2^d	*H-2*^{b}	DBA/2	C57BL (0)
H-2^d	*H-2*^{b}	C57BL/10 (*H-2*^{d})	C57BL/10 (13)
H-2^k	*H-2*^{d}	AKR	BALB/c (0), DBA/2 (0)
H-2^k	*H-2*^{d}	C3Hf	BALB/c (50), DBA/2 (20)
H-2^k	*H-2*^{b}	CBA/Jax	C57BL (0)

*Percent calculated on 14-day survivors.

Discussion

Histocompatibility genes.—The basic genetic principles of tissue transplantation have been clearly defined, and they follow simple Mendelian laws of inheritance (*18*, *23*). The same principles apply to the transplantation of normal as well as neoplastic tissue and should also apply to the transplantation of marrow in the irradiated host. The nonspecific

paralysis of the host's immune system by irradiation adds a complicating factor which has resulted in the controversy as to whether the secondary phase of the irradiation syndrome is an immune response of the marrow-graft against the irradiated host (*9–15*), of the host against the graft as summarized by Makinodan *et al.* (*21*), or possibly a combination of both. The histocompatibility genes play an important part in this reaction, regardless of where the reaction is initiated.

Counce *et al.* (*20*) studied the influence of the *H-1* and *H-3* genes on tissue homografts. When the same two pairs of coisogenic lines were tested for their effect on tumor and skin homografts, it was found that the *H-3* locus was only a minor obstacle, with a clear-cut increase in survival time of the homotransplants. From gross observation, the marrow graft and the irradiated host appear not to be influenced by a gene difference at the *H-3* locus. Histological evidence of a reaction has not been determined since too few animals of this group have reached the moribund stage and been autopsied.

In the *H-1* locus, represented by C3Hf and C3H.K, there was individual variation in the expression of the reaction in the irradiated hosts. Some animals showed a reaction which was seldom lethal, while others gave no gross indication of a reaction. Counce *et al.* report variation in survival of skin homografts. Upon further investigation, this variation has been attributed to technical difficulties of the skin-grafting technique (*24*). An explanation for the variability among irradiated hosts is not immediately forthcoming. Pooled C3H.K marrow was used to treat irradiated C3Hf mice; therefore the variability may not be attributed to residual heterozygosity in the C3H.K coisogenic line. All mice in an experimental group received a uniform inoculum of marrow cells. It appears likely that this variability may be a threshold effect: the reaction expressed in some individuals and not in others.

The *H-2* locus is a strong histocompatibility locus, and a difference of phenotype at this locus will prevent the progressive growth of nearly all tumor homotransplants and cause the rapid rejection of skin homografts. In the testing of a large number of inbred-strain combinations in this laboratory, no *inbred-strain* combination has been observed in which good long-term survival was successfully achieved when the strains were of different *H-2* phenotypes. The percentage of survival varied with the strain combinations employed. When the available data on different irradiated F_1 hybrid mice treated with parental-strain marrow (*e.g.*, *13*, *16*) are compared, variations are observed which are not found when comparing data on most inbred-strain combinations.

Between inbred strains sharing the same *H-2* phenotype, the long-term survival was good within the *H-2*^{b} and *H-2*^{d} groups. All strain combinations tested within each of these two groups appeared compatible, *i.e.*, there was good long-term survival. Attention should be drawn to the fact that within the *H-2*^{k} group there is a variability of response between different strain combinations. Preliminary data available on strain combinations of the *H-2*^{k} group indicate varying degrees of reaction, from

no reaction to a severe, lethal reaction (unpublished data). Since the status of the *H-2*^{k} group is still largely undefined and the number of animals in each experimental combination is relatively small, no attempt will be made at this time to explain the variability observed in some strain combinations of this group when strain barriers are crossed.

The additive effect of the *H-2* genes in the heterozygous F_1 hybrid can be demonstrated by mating an *H-2*^{d} with an *H-2*^{k} mouse, which gives a genotype of *H-2*^{d}/*H-2*^{k}. The phenotype of *H-2*^{a}, formerly called *H-2*^{dk}, is A.C.D.E.F.H.J.K.M.N.Y. and the phenotype of the hybrid is a.C.D.-E^d.F.H.J.k.M.N.y./A.C."D^k."E.f.H.j.K.m.n.Y. (*17*, P. A. Gorer, personal communication). Thus the hybrid carries all the known antigens of *H-2*^{a}, and will support a strain A tumor (*25*, *26*). The hybrid has a number of antigens not present in strain A mice, such as, "D^k,"[4] E^d, f, etc., and a hybrid tumor will not usually take, and grow progressively, in strain A mice. Although not all these factors have been demonstrated serologically, Gorer has shown that anti-"D^k" is a potent cytotoxin. Anti-E^d (*27*) and another factor which could be anti-"D^k" or anti-f are both strong agglutinins under suitable conditions. (D. B. Amos, personal communication.)

When strain A mice are recipients of marrow from the *H-2*^{d}/*H-2*^{k} hybrids (ST $\times$ ST.T6)F_1 or (AKR $\times$ DBA/2)F_1,[5] all mice that survive 14 days survive over 90 days. This may also occur in (C3Hf $\times$ DBA/2)F_1 hybrids, but the available data are insufficient to make such a positive statement at this time. The only reciprocal test made to date is the (AKR $\times$ DBA/2)F_1 host, with strain A marrow. In this combination there is a reaction. Of 40 mice treated, one died within the first 14 days, but only 50 percent of the treated mice survived over 90 days.

These data give further support for the concept of an immune response from a bone marrow graft. Presumably the hybrid animal cannot produce antibody against the strain A tumor, and likewise the hybrid marrow does not react against the tissues of the *H-2*^{a} host to produce *H-2* antibodies, though weaker non-*H-2* antibodies could be formed. Conversely, just as *H-2*^{a} reacts against the unshared antigens of the hybrid, as illustrated by the reaction against F_1 hybrid tumor tissue, strain A marrow reacts to produce "secondary disease" in the irradiated F_1 hybrid. It would be of interest to know whether a similar reaction would occur if strain A marrow were used in the (ST $\times$ ST.T6)F_1 hybrid. A reaction would be expected with strain A marrow in the (C3Hf $\times$ DBA/2)F_1 hybrid, since the "D^k" factor can be demonstrated with strain A anti-C3H serum as well as with the isogenic resistant lines of AKR. (P. A. Gorer, personal communication.)

The gene-dosage phenomenon.—The additive effect of multiple doses of a single gene is a well-established genetic phenomenon. A recent review (*28*) of the genetic aspects of tissue transplantation will point up the fact that this is not a new concept. The gene-dosage concept applied to the

[4] Dr. Gorer uses the symbol "D^k," since it is not certain how many antigens are concerned in this reaction.
[5] Unpublished data.

data on irradiated F_1 hybrid mice treated with parental-strain marrow could explain why these responses are different than in different inbred-strain combinations. In the latter, there was a severe and lethal reaction when the strains were of different *H-2* phenotypes, yet in some F_1 hybrid mice treated with parental-strain marrow there was good long-term survival after a mild reaction, *e.g.*, a 90-day survival of 85 percent was observed in a combination which was heterozygous at the *H-2* locus (*13*).

The inbred strains are homozygous at the *H-2* locus, *i.e.*, each animal has two identical genes at this locus. In the F_1 hybrid mice, which are heterozygous at the *H-2* locus, there are two different, partially dominant, genes, each showing some degree of expression. The gene-dosage relationship of the grafted marrow to the irradiated host is a double dose of one gene in the graft and a single dose of two different genes in the host. The hybrid host presents half the antigenic differences as represented by the single incompatible gene, in contrast with the antigenic difference presented by the double gene dosage in two inbred strains. In the hybrid, a single gene difference may not be sufficient to reach the threshold necessary for a reaction, while the two-gene difference in the inbred strains may be sufficient to surpass this threshold and to cause a lethal reaction.

There are two other sets of data which appear to be at variance with the accumulated data on inbred-strain combinations, yet these data can be explained by the gene-dosage hypothesis. The data reported by van Bekkum *et al.* (*16*) on C57BL and CBA mice will be considered later as an indication of a possible threshold effect. The other strain combination of C3H and 101 can also be used for illustration. These strains were found to be incompatible, regardless of which strain is used as marrow donor or irradiated recipient, but in the irradiated F_1 hybrid, parental-strain marrow was found to be compatible (G. E. Cosgrove, personal communication). Since both strains are of the $H\text{-}2^k$ phenotype, the presence of a reaction between the inbred strains would indicate undefined, antigenic differences at the *H-2* locus, or antigenic differences which are not *H-2*. These results are not at variance with the proposed hypothesis, since the same dosage and threshold effect could apply to the presence of a reaction between the inbred strains and the absence of a reaction in the hybrid, which resulted from antigenic differences at the *H-2* locus or unshared antigens not governed by the *H-2* locus. This situation appears to be comparable with that discussed for the $H\text{-}2^d/H\text{-}2^k$ hybrid. Experiments are in progress which may define more clearly the limitations involved with different strain combinations within the $H\text{-}2^k$ group.

Two factors bring the *H-2* genes in line with the human blood groups: 1) The *H-2* genes are partially dominant and are expressed in the heterozygote as illustrated in the $H\text{-}2^d/H\text{-}2^k$ hybrid. 2) The *H-2* locus regulates a series of erythrocyte antigens. In the human blood groups, a gene-dosage effect may be illustrated by the *MN*. and *Ss* of the *MNSs* system, Rh, Kell, and Duffy antigens (*see* references cited in *29*). Since gene action is, in part, a result of the interaction between genes and their environment, it is not surprising that Cohen (*30*) found other factors,

in addition to gene dosage, which influenced the hemagglutination tests in rabbits; a fact, he points out, which has also been demonstrated in human blood groups.

Although the gene-dosage effect is a well-established genetic phenomenon and has been studied extensively in *Drosophila*, the proof of such a phenomenon in the mouse would be difficult. For example, in *Drosophila*, the gene-dosage effect was delineated for the bobbed alleles (*31*) or *cubitus interruptus* alleles (*32*) by building stocks which carried 1, 2, 3, and 4 doses of the gene which were tested within a single environmental background. To build up multiple gene dosages within the coisogenic lines in the mouse would be a long and arduous task, if indeed it is even possible, yet this would be a method of testing gene dosage in a nearly uniform environment.

A possible threshold effect.—F_1 hybrids treated with parental-strain marrow which produced good long-term survival have been observed both in this laboratory and elsewhere (*13*, *16*, *33*). Schwartz *et al.* (*33*) observed in one of these combinations that splenic tissue obtained from one parental strain elicited a reaction that was fatal to the irradiated F_1 hybrid host. With this lead, several different types of combinations, in which homologous marrow appeared to be compatible, were tested to determine whether splenic tissue would elicit a reaction. In combinations in which there was a mild reaction with homologous marrow, a severe and lethal reaction followed the inoculation of splenic tissue—a situation similar to that of Schwartz. Splenic tissue did not elicit a reaction in all combinations. In (BALB/c $\times$ DBA/2)F_1 hybrid mice treated with DBA/2 hematopoietic tissue, either marrow or splenic tissue produced equally good long-term survival. Inbred-strain combinations such as C57BL/10(*H-2*^{d}) and DBA/2 treated with either marrow or splenic tissue had equally good long-term survival. In the latter two combinations, the inbred strains involved are all of the *H-2*^{d} phenotype and can be used interchangeably as irradiated hosts and donors without apparent ill effects (unpublished data).

There is another strain combination reported by van Bekkum *et al.* (*16*), which will serve to illustrate the possible existence of a threshold effect. Strains C57BL and CBA were found to be incompatible when inbred strains were used, but there was only a slightly reduced survival when marrow from strain C57BL was introduced into the irradiated F_1 hybrid. No decreased survival was observed with CBA marrow. Since all the sublines of CBA thus far tested are of the *H-2*^{k} phenotype and the sublines of C57BL are either *H-2*^{b} or *H-2*^{d} (P. A. Gorer, personal communication), it may be assumed with some degree of justification that the strains used in these experiments are of different *H-2* genotypes. These results may be explained on the basis of the proposed gene-dosage hypothesis, although no reduced long-term survival indicates that a reaction, if present, was not lethal, and does not in itself indicate the complete absence of a reaction. The antigenic differences found in the F_1 hybrid host treated with parental-strain marrow may fall at or below the threshold necessary for the in-

duction of a lethal or even discernible reaction. This may account for the variability found in the comparison of different F_1 hybrid mice treated with parental-strain marrow. For example, there was a mild reaction observed in the (C57BL × A)F_1 hybrids treated with marrow of either parental strain; in contrast, a lethal reaction was found in (C57BL × DBA/2)F_1 hybrids treated with C57BL marrow (*13*).

In the experiments of van Bekkum *et al.* (*34*), a threshold effect may be demonstrated by the addition of lymphoid tissue to the bone marrow, which in the C57BL-CBA combination produces a reaction. In mice treated with marrow, the animals survive the reaction if the antibody peak is below a critical threshold. In the animals in which the critical threshold is not reached, recovery occurs when the immunological reaction fatigues, or a form of "adaptation" is achieved, or as a result of some, as yet, unknown phenomenon.

Conclusion

Although the gene-dosage hypothesis appears to be a plausible explanation for the genetic data available to date, further investigation is necessary to determine whether these data can actually be explained on the basis of gene dosage or whether some as yet unknown phenomena are involved. Undoubtedly, the resolution of the dilemma will come only when there is an unequivocal serological demonstration of the antibodies involved in these reactions.

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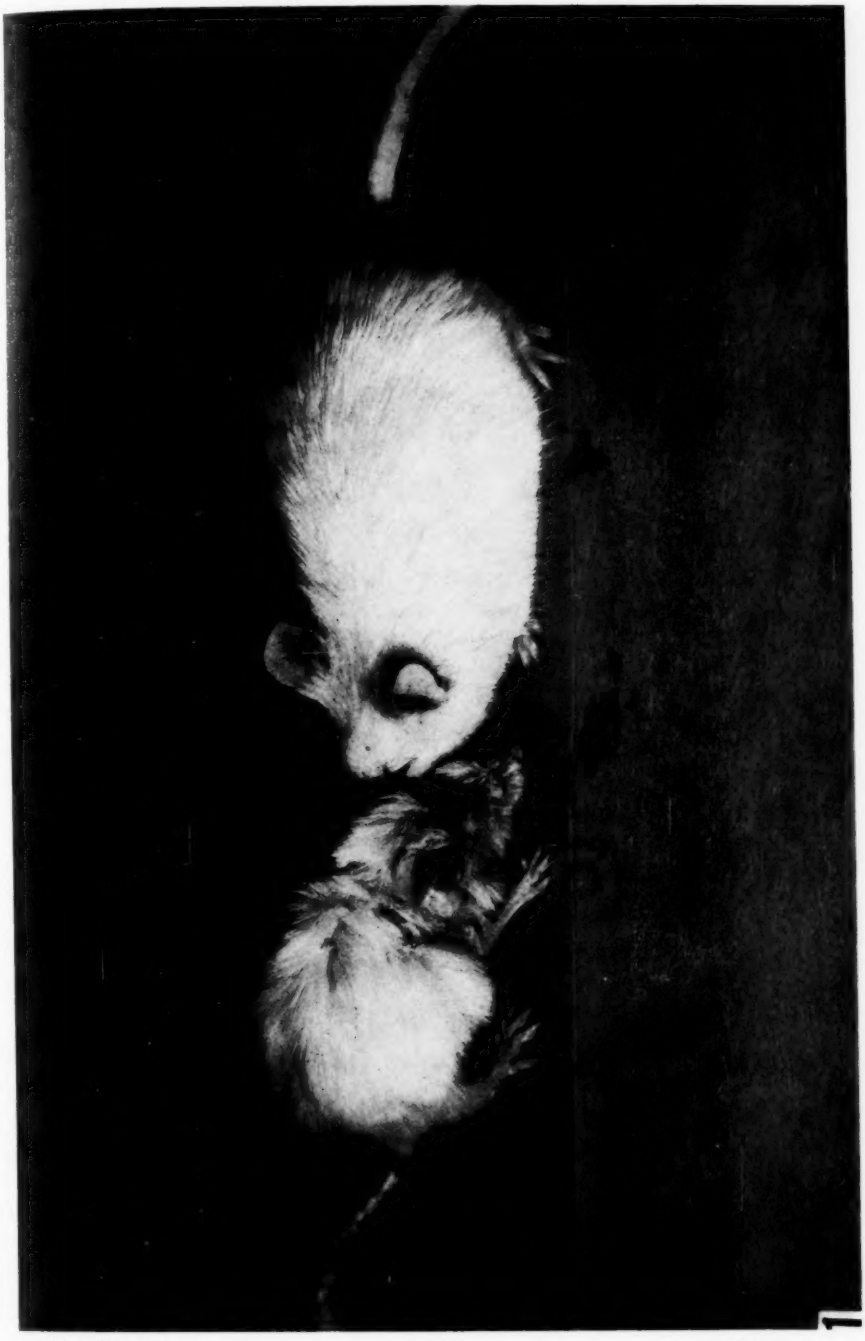
PLATE 21

FIGURE 1.—Strain ST mice 72 days after exposure to 800 r total-body X radiation. The normal appearance of the mouse which received isologous marrow is contrasted with a typical reaction observed in a mouse receiving coisologous marrow. This mouse died 77 days after treatment.



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Mitotic Distribution of Feulgen Material in Three Ascites Tumors [1]

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Summary

The Feulgen absorbances of each of the two chromosome sets of anaphase and telophase nuclei were measured with the universal microspectrophotometer. Significant differences between the Feulgen absorbances of the daughter nuclei were found in 41.5 percent of nuclei from Ehrlich ascites tumor, in 16.7 percent from L1210 lymphoid leukemia, and in 18.7 percent from MC1M tumor. The possible significance of unequal nuclear division in producing aneuploidy was briefly discussed.—**J. Nat. Cancer Inst. 22: 243–257, 1959.**

The development of Feulgen microspectrophotometry has made it possible to analyze the amount of deoxyribonucleic acid (DNA) during the mitotic process in individual nuclei. Investigations concern the time of DNA synthesis, the DNA content in different phases, and the apportionment of the DNA during mitosis. The most prevalent concept of the synthesis of DNA, although not universally accepted, is that it takes place during interkinesis—before the onset of mitosis, and that the DNA is distributed *equally* by the mitotic process (*1–8*). A fairly good correlation between the chromosome number and Feulgen absorption has been established (*9–12*), though the DNA values can disclose polyploid cells not apparent from chromosome counts (*13*).

It is well established that tumor cells are characterized by greater mitotic instability and higher frequency of mitotic disturbances than normal cells. Among other mitotic abnormalities, the unequal distribution of chromosomes is frequently mentioned by authors without a quantitative analysis of the phenomenon (*14–19*). The unequal distribution of chromatic material is difficult to investigate by ordinary cytological methods, *viz.*, chromosome counting, but can be studied by Feulgen microspectrophotometry.

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[3] I wish to thank Dr. Klein and Dr. Bayreuther for their valuable criticisms, Dr. Révész for the tumors, and G. Dallner for his valuable help.

Microspectrophotometry of mitotic figures is difficult, particularly because of the irregular shape of the object. However, with the scanning-line system of Caspersson the distributional error is significantly reduced (*20*).

The Feulgen density of each of the 2 chromosome sets of late anaphase and telophase nuclei was measured in 3 ascites tumors. It was found that an unequal distribution of DNA is a frequent occurrence during anaphase and telophase. This may be of significance in producing aneuploidy in tumor cells.

Materials and Methods

Tumors.—The following tumors were used: Ehrlich ascites tumor (designated ELT/Stockholm), 11-day-old tumor; L1210 lymphoid leukemia, 4-day-old tumor, 279th transfer generation; MC1M sarcoma, 6-day-old tumor, 207th transfer generation (*14*, *21–23*).

Feulgen reaction.—The ascitic fluid was diluted with cold Ringer's solution and smeared. The smears were dried for 30 minutes at room temperature. After 40 minutes' fixation in acetic acid:ethanol, 1:9, they were rinsed in distilled water and cold N HCl. The smears were hydrolyzed for 12 minutes in N HCl at 60° C. and placed in Schiff's reagent (deTomasi) for 90 minutes. Finally, the slides were rinsed in SO_2-water, dehydrated, and mounted in Canada balsam.

The smears of thymocyte suspension were made in the following way: Adult mice of both sexes were quickly killed without anesthesia, and the thymus was removed immediately. After removal of the connective tissue, the thymus was cut into small pieces and shaken in cold Ringer's solution. Small drops of this suspension were smeared on slides, dried at room temperature, treated, stained, and measured in the same way as the tumor tissue. The data representing the frequency distribution of Feulgen absorbance of thymocytes were pooled from several measurements that were made at different times on various suspensions.

Measurements.—Caspersson's universal microspectrophotometer (*24*) was used with a 5× ocular, Zeiss apochromate 90× objective and a condenser of N. A. = 0.2. Measurements were made at 546 mμ. The distance between 2 successive scanned bands was 1 μ. The scanned strip was 0.31 μ wide. The universal spectrophotometer is coupled with a function transformer and integrater, which makes possible the automatized determination of the total extinction of the scanned area. Thus the extinction of Feulgen absorbance of the 2 separated parts of anaphase and telophase figures could be determined.

The cells measured were chosen by the following criteria: The cell had to be in late anaphase or telophase and in a favorable position for measurement, *i.e.*, free of contact with other cells. The two absorbing masses had to be distinctly separated from each other by at least 2 or 3 μ. Great care was taken to distinguish late telophase from 2 closely situated cells in the resting stage. Furthermore, no cells that manifested gross mitotic abnormalities were measured.

All measured cells were sketched and some were photographed (figs. 1 and 2). The 2 parts of dividing cells are designated "daughter" cells. The measured extinction maximums were below 1.0. The absorbance of nonhydrolyzed cells was hardly measurable and therefore could be neglected.

Statistical methods.—It is reasonable to assume that each of a given pair of daughter nuclei would be subjected to nearly identical conditions during the Feulgen staining procedure because of their close spatial relationship. For the same reason, any systematic optical errors would not be expected to interfere significantly with the interpretation of the data. Furthermore, the orientation of chromosomes with respect to the optical axis does not seem to influence the exactness of measurements.

To investigate the distribution of the Feulgen material in the mitotic figure the first task was to determine the errors of measurement. Differences found in the Feulgen absorbances of the 2 daughter cells might be due to chance or to a real difference between the 2 nuclei, depending on errors of measurement.

Duplicate measurements were made in order to estimate the random measuring errors. For all 3 tumors the random measuring errors were calculated from duplicate measurements and these were applied to the 3 individual groups. The method was as follows: The total extinctions of the 2 daughter cells (fig. 2, *a* and *b*) were measured. The microscope stage, together with the slide, was then rotated 1 to 5 degrees, and *a* and *b* were measured again in this second position. In this way two duplicates were obtained, one for *a* and one for *b*. The standard deviation of the differences between the duplicates was calculated. The observational error on each occasion = standard deviation/$\sqrt{2}$, and the standard error of difference = $\sqrt{\text{S.E.}_1^2 + \text{S.E.}_2^2} = \text{S.E.}\sqrt{2}$, because S.E. is common for *a* and *b*. When two measurements were obtained from the same cell and the difference was calculated from the means, the standard error of difference = standard deviation.

With the S.E. of the difference, the differences significant at various levels ($P = 0.1$ to 0.001), as calculated from the equation $t = \frac{\text{difference}}{\text{S.E.}}$ or difference = t S.E., were determined. It should be noted that a single measurement is equivalent to the mean of one observation. By the calculation of differences the number of duplicate measurements was taken as the number of degrees of freedom. By rotating the microscope stage [4] between the 2 measurements, the reliability of the determination of the error was thought to be enhanced. When there are parallel determinations the difference which is significant on a given level is less than the significant difference as determined from 1 measurement by a factor of $\frac{1}{\sqrt{2}}$. The differences between the daughter cells in all the measured anaphase and telophase figures were determined by this statistical analysis and grouped according to the probability of the significance. To demonstrate the method, the analysis of one group is described in detail (table 1).

[4] Suggested by Dr. Bahr.

TABLE 1.—*Analysis of duplicate measurements in Ehrlich ascites tumor**

Number of cell	Duplicates (*arbitrary units*)	Difference between duplicates	*n*
3)*a*	266. 0		
	238. 9	28. 1	1
b	298. 3		
	311. 6	13. 3	2
14)*a*	314. 0		
	335. 2	21. 2	3
b	253. 9		
	321. 2	67. 3	4
15)*a*	157. 1		
	189. 8	32. 7	5
b	245. 2		
	224. 3	20. 9	6
16)*a*	333. 6		
	361. 1	27. 5	7
b	383. 8		
	412. 2	28. 4	8
17)*a*	400. 5		
	408. 5	8. 0	9
b	351. 2		
	345. 8	5. 4	10
18)*a*	221. 8		
	243. 2	21. 4	11
b	346. 1		
	338. 9	7. 2	12
19)*a*	115. 1		
	131. 5	16. 4	13
b	123. 2		
	131. 4	8. 2	14
20)*a*	127. 9		
	131. 0	3. 1	15
b	157. 5		
	152. 5	5. 0	16
21(*a*	135. 0		
	126. 8	8. 2	17
b	109. 1		
	112. 7	3. 6	18
22)*a*	108. 2		
	100. 8	7. 4	19
b	147. 1		
	136. 0	11. 1	20
24)*a*	148. 0		
	147. 5	0. 5	21

See footnotes at end of table.

TABLE 1.—*Analysis of duplicate measurements in Ehrlich ascites tumor**—Continued

Number of cell	Duplicates (*arbitrary units*)	Difference between duplicates	*n*
24)*b*	125. 0 119. 9	5. 1	22
25)*a*	115. 1 121. 8	6. 7	23
b	121. 0 108. 9	12. 1	24
26)*a*	125. 4 126. 0	0. 6	25
b	130. 1 132. 0	1. 9	26
27)*a*	160. 2 156. 5	3. 7	27
b	151. 7 133. 0	18. 7	28

*Variance $= \frac{10830.99}{2x\ 28} = 193.4$; standard deviation $= \sqrt{193.4} = 13.9$.

Coefficient of variation = 6.8.

Observational error on each occasion $= \frac{13.9}{\sqrt{2}} = 9.83$.

Standard error of observation = 13.9.

Standard error of difference = 13.9. $\sqrt{2} = 19.65$ (one measurement).

Standard error of difference = 13.9 (duplicate measurement).

$t = \frac{\text{diff.}}{\text{S. E.}}$; and difference = t 19.65 (one measurement).
difference = t 13.9 (duplicate measurement).

Degree of freedom = n = 28

if	$P = 0.1$	$P = 0.05$	$P = 0.01$	than
	$t = 1.70$	2.05	2.76	and the differences

which are significant on the different level are:

33.40	40.28	54.23 (one measurement)
23.63	28.49	38.36 (duplicate).

The grouping of mitotic cells was made according to the calculated differences. If the difference between the Feulgen absorbance of the daughter cells was, *e.g.*, 45.0 (single measurement), the difference is significant on the 5 percent level ($P = <0.05$).

The random measuring errors calculated from 28, 24, and 22 duplicate measurements of Ehrlich, L1210, and MC1M tumors, respectively, were 6.8, 7.0, and 4.8 percent (coefficient of variation).

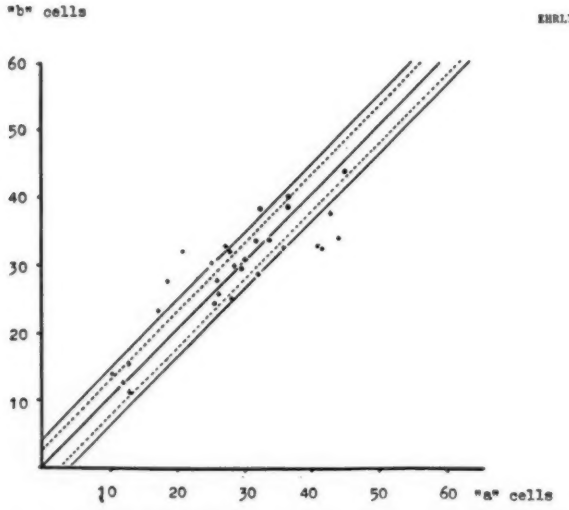
The error determined from duplicate measurements also was checked with another error calculated from parallel determinations. The absorbance of 1 nucleus in telophase was measured 15 times. In this case the cell was not rotated but shifted in the direction of Y axis and brought back into focus before each measurement. With the shifting of the cell position, the path of the scanning lines relative to the cell was changed. The standard errors as the percentage of the means calculated

from parallels were 4.2 and 4.9 percent for *a* and *b* and 7.0 percent for the whole cell.

Results

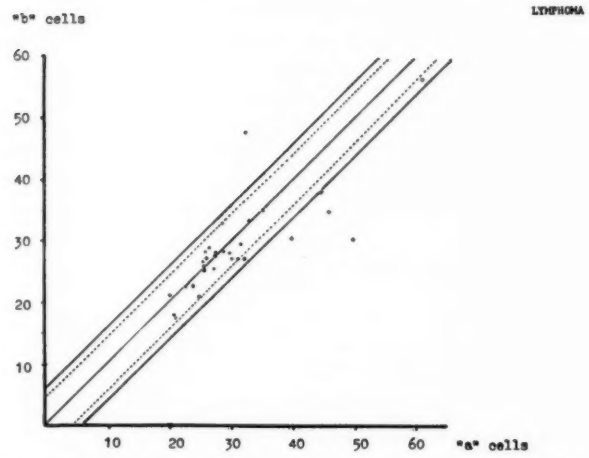
The results of the measurements are shown in text-figures 1 to 7.

Scatter diagrams (text-figs. 1, 2, and 3) represent the correlation of the Feulgen absorbances of the daughter cells. The position of each point on the diagram is determined by the Feulgen absorbances of the daughter cells and so the diagram makes it possible to read the original data of the measurements without tables. Points and crosses within the lines on the diagram represent dividing cells in which the Feulgen absorbances of the daughter cells do not differ significantly ($P>0.05$). Dotted lines and crosses are used for duplicate measurements and solid lines and dots for single measurements. The widths of the bands were calculated from duplicate measurements, described in "Methods," in each group. The straight line from the origin represents the theoretically perfect positive correlation that would exist if the Feulgen material were apportioned equally by the division.



TEXT-FIGURE 1.—Ehrlich ascites tumor. Feulgen absorbances of nuclei in anaphase and telophase. The values on the ordinate and abscissa represent the Feulgen absorbances, in arbitrary units, of daughter-cell pairs.

Text-figures 4, 5, and 6 are histograms showing the frequency distribution of the Feulgen absorbances of the daughter cells. In the histogram each daughter cell represents an individual value. The histograms do not provide a comparison between Feulgen absorbance of daughter-cell pairs, but they give an idea of the frequency distribution of Feulgen material in the dividing cells.

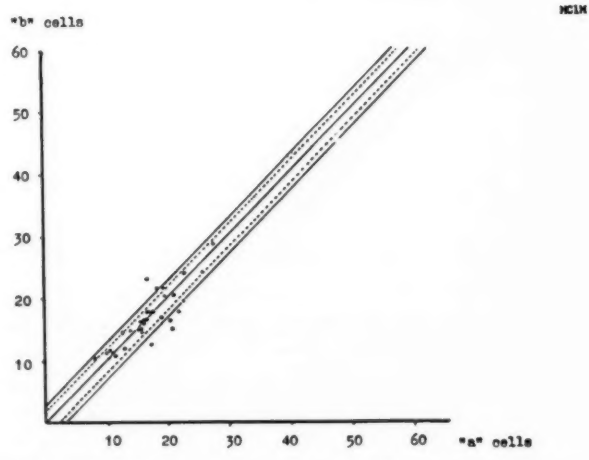


TEXT-FIGURE 2.—Lymphoid leukemia L1210. Feulgen absorbances of nuclei in anaphase and telophase. The values on the ordinate and abscissa represent the Feulgen absorbances, in arbitrary units, of daughter-cell pairs.

The diagram of text-figure 7 represents the frequency distribution of Feulgen absorbance of normal cells, measured on suspensions of thymocytes by the same technique.

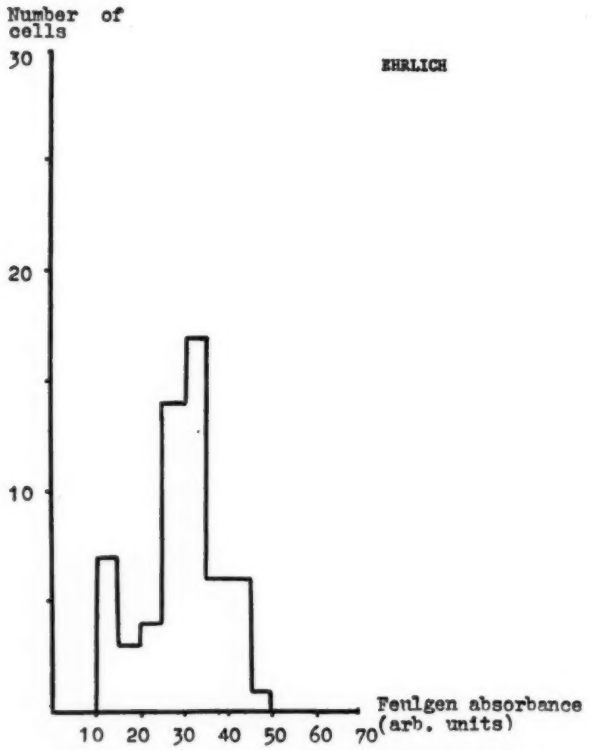
The results of the measurements can be summarized in the following:

Ehrlich ascites tumor.—The Feulgen absorbance of 29 nuclei in anaphase and telophase was measured. Unequal distribution of the Feulgen material between the daughter cells was found most frequently in the Ehrlich



TEXT-FIGURE 3.—MC1M sarcoma. Feulgen absorbances of nuclei in anaphase and telophase. The values on the ordinate and abscissa represent the Feulgen absorbances, in arbitrary units, of daughter-cell pairs.

ascites tumor. The difference between the Feulgen absorbances of the daughter cells was significant at the 5 percent level in 3 cells, and at the 1 percent level in 12 cells; in 8 of these the probability that the difference was due to chance was less than 0.1 percent ($P<0.001$). To summarize: In 41.5 percent of the measured anaphase and telophase figures a significantly unequal distribution of Feulgen material (DNA) was found. The Ehrlich ascites tumor showed the highest frequency of unequal mitosis and also the largest differences between the Feulgen absorbances of the



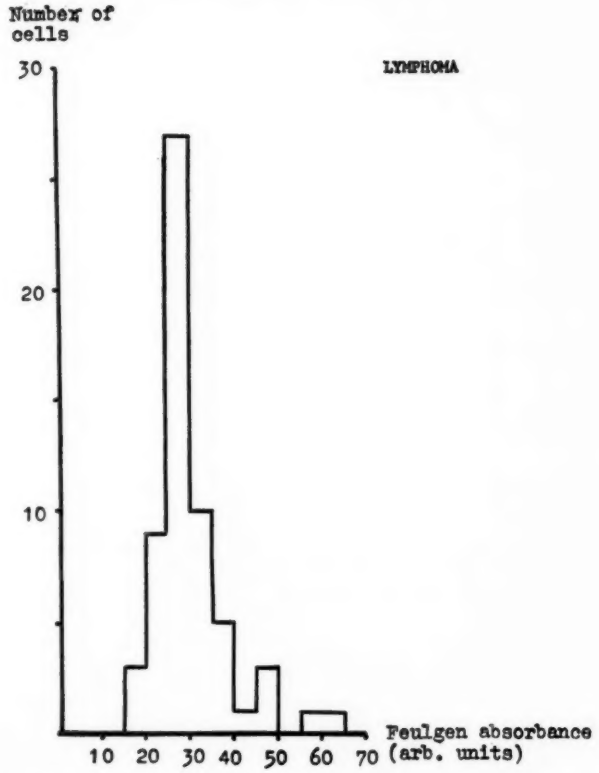
TEXT-FIGURE 4.—Ehrlich ascites. Frequency distribution of Feulgen absorbances of daughter cells.

2 daughter cells. The difference was 10.6 percent of the mean of the daughter-cell absorbances.

L1210 lymphoid leukemia.—Among the measured 30 dividing nuclei, 5 were found to have a significantly unequal distribution of Feulgen material. In 2 the difference was significant at the 1.0 percent level and in 3 of these pairs at the 0.1 percent level, *i.e.*, 16.7 percent of the measured cells showed unequal mitosis but 83.3 percent did not show demonstrable differences on the basis of the method outlined. The mean difference was also less

than in the Ehrlich tumor, *i.e.*, 5.5 percent of the mean of the Feulgen absorbance of the daughter cells.

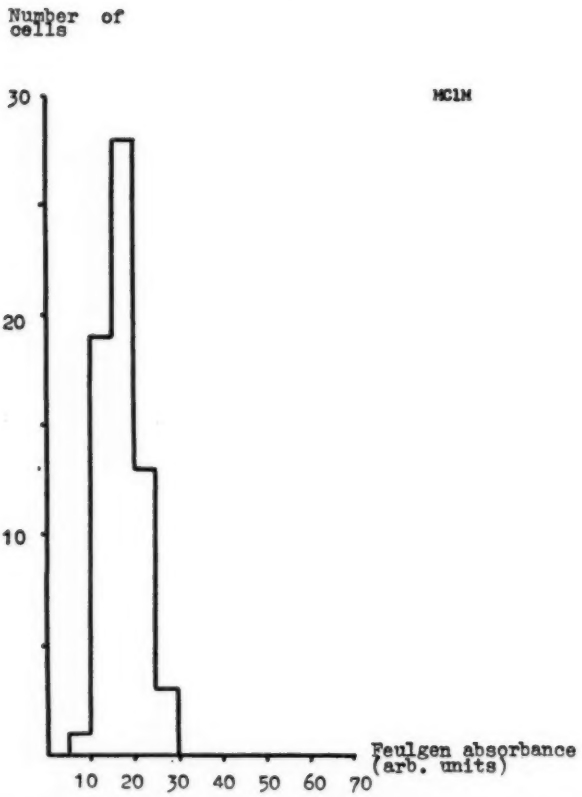
MC1M sarcoma.—Thirty-two nuclei in anaphase and telophase were measured. In 6 cells a significant difference in Feulgen absorbance of the 2 daughter cells ($P<0.01$) was found, and in 3 of these pairs P was <0.001. The frequency of significantly unequal mitosis was 18.7 percent, and the mean of differences was 4.7 percent.



TEXT-FIGURE 5.—Lymphoid leukemia L1210. Frequency distribution of Feulgen absorbances of daughter cells.

The anaphase and telophase values show differences in Feulgen absorbance. The tendency for unequal mitosis to occur more frequently in cells with higher DNA content (probably higher chromosome number) is clearly suggested by the data of MC1M sarcoma and L1210. In the Ehrlich ascites tumor the Feulgen values of the unequal mitoses were more frequently scattered. Whether this is an indication of a mixed cell population will be discussed later.

There is fairly good agreement between the variation in chromosome number of Ehrlich and MC1M tumors determined by Levan and Hauschka

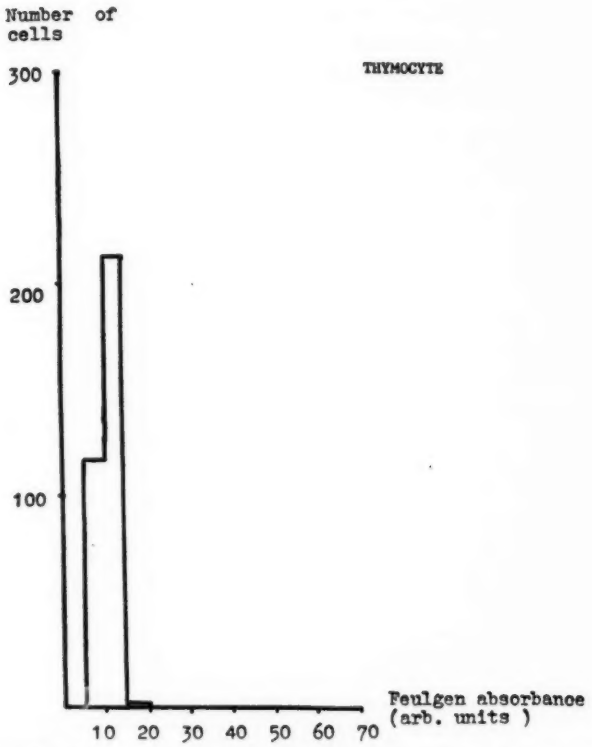


TEXT-FIGURE 6.—MC1M sarcoma. Frequency distribution of Feulgen absorbances of daughter cells.

(*23*) and the variation in Feulgen absorbance in these tumors ascertained by us. According to the authors mentioned, the Ehrlich ascites tumor used in this study has a stemline number of 80 and is near-tetraploid, while the MC1M is hypotetraploid, with a stemline number of 65. The mean Feulgen absorbance of the daughter cells was found to be 29.5 in the Ehrlich ascites tumor, and 16.7 in MC1M tumor. The diploid value of the mouse thymocyte nuclei, by the same method, is 10.7 (coefficient of variation: 14.7%), and therefore the tetraploid value is 21.4. These values correspond to hypertetraploid and hypotetraploid values, respectively. We have found no data in the literature for the distribution of the chromosome number of the L1210 lymphoid leukemia.

Discussion

In 1890, Hansemann (*25*) clearly and unambiguously described the occurrence of the "asymetrische Mitose" in human tumors. He stated that there did not exist any carcinoma without unequal mitosis. The present



TEXT-FIGURE 7.—Frequency distribution of Feulgen absorbances of mouse thymocytes.

work translates Hansemann's conclusions into modern terminology and puts it on a quantitative basis.

Tumors consist of genetically heterogeneous cell populations. This is expressed as a variation in chromosome number around a modal number, the so-called stemline number that is usually aneuploid. Among the chromosomal mechanisms responsible for the variation around the stemline number [lagging of chromosomes, multipolar spindle, the different types of endoreduplication, chromosome elimination, and anaphasic nondisjunction (*15*, *17–19*, *26–28*)], the occurrence of unequal mitosis is frequently mentioned. In the present study, the unequal distribution of DNA (Feulgen material) during the mitotic process is recorded. The type of cytological mechanisms involved in the unequal distribution of DNA is not known, but it may be assumed that this unequal distribution of DNA reveals itself also in the chromosome numbers of the daughter cells. Unequal distribution of the chromatic material could be a simple explanation of the formation of most of the aneuploid cells, since it can lead to loss of chromosomes in one of the daughter cells and to the origin of hypodiploid cells as described recently in human tumors (*29*). The mechanism of origin of cells with various chromosome numbers which

predominate in different tumors probably varies, but unequal mitosis (unequal distribution of DNA) seems to be more important than lagging of chromosomes, multipolar spindle,[5] etc.

Unequal mitosis seems to occur more frequently in cells with higher DNA content (with greater amount of Feulgen material), which indicates that the greater the chromosome number, the more frequent the disturbances in the chromosomal mechanisms. In L1210 the peak of the Feulgen values of the daughter cells lies between 25 to 30 (arbitrary units), while all the unequal mitosis values have higher absorbances. In MC1M tumor the most frequent Feulgen values are between 10 to 15 and 15 to 20. The unequal mitoses are divided equally between the categories of 15 to 20 and 20 to 25, which again indicates the tendency, mentioned before, for the frequency of unequal division to be higher in cells with higher Feulgen values. The Feulgen values of daughter cells in Ehrlich ascites tumor show 2 peaks. They are between 10 to 15 and 25 to 35. The values of unequal mitosis scatter too much to suggest a definite tendency. However, from the frequency distribution of Feulgen values and the scatter diagrams, one may speculate that these data give some indication of a genetic, mixed cell population. If this is so, the unequal mitosis values around 20 would represent the higher polyploid variant of one population with a modal number around 15, and the unequal mitosis values between 30 to 45 would represent variants of another cell population with a modal number of 30 to 40.

Among the tumors investigated the highest percentage of unequal division occurred in Ehrlich ascites tumor. The tumor used was 11 days old; therefore, it is not strictly comparable with the other 2 tumors, which were 4 to 6 days of age. According to Koller (*15*), the mitotic disturbances are connected with nutritive factors. In an 11-day-old ascites tumor these nutritional factors may be more unfavorable for the cells. In L1210 lymphoid leukemia the percentage of unequal division was the smallest of the 3 tumors; on the other hand, the relative stability of chromosome mechanisms of mouse leukemias and lymphomas is well known.

We should like to mention that Hsu and Moorhead (*27*) observed unequal mitosis in HeLa cells with cinematography. However, though our study shows variation of Feulgen densities of nuclei in telophase, Richards and Richards *et al.* (*11*, *30*) did not find such a variation of telophase DNA values in Ehrlich and Krebs tumors, which one would expect because of the aneuploidy of interphases and telophases. These differences between the observations of Richards and those presented are probably real in Ehrlich tumor and not because of divergent photometric techniques. The discrepancy could be caused by biological variations (tumor stock, age of the tumor, etc.) or by the variation in the Feulgen techniques.

According to Levan (*27*), normal tissue does not show major variation of chromosome number. On this basis, the occurrence of unequal divi-

[5] One tripolar mitosis was found and measured in Ehrlich ascites tumor. The extinction values of the 3 Feulgen masses were: 15.8, 9.97, and 12.1.

sion in normal tissues would not be expected. Patau and Swift (*31*) have found in root meristem that the mean DNA content per set of chromatids in late interphase, prophase, and telophase is identical. However, the number of nuclei measured is too small to permit conclusions of statistical significance. We tried to measure the Feulgen absorbance of telophase and anaphase figures of normal rat cells of the Lieberkühn crypts. However, with sections, the exactness of the type of measurement required with this technique became so questionable that definite conclusions could not be reached.

We are aware that the observations described need further clarification. The correct evaluation of the significance of unequal nuclear division in a given tumor depends on serial measurements at various times and under various circumstances, just as repeated cytological analysis is necessary to give a true picture of the chromosome pattern of the tumor.

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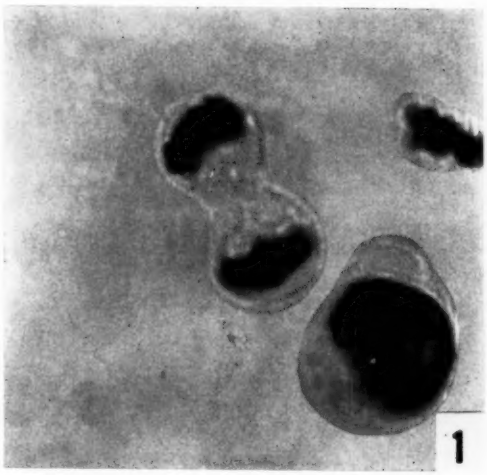
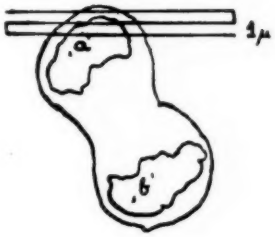
PLATE 22

FIGURE 1.—Telephase nucleus from Ehrlich ascites tumor.

FIGURE 2.—Sketch illustrating pattern of the scanning.





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Emotional and Other Selected Characteristics of Cigarette Smokers and Nonsmokers as Related to Epidemiological Studies of Lung Cancer and Other Diseases [1,2]

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Summary

In 1956 a probability sample of the adult populations of Buffalo, New York, was interviewed to determine whether cigarette smokers differed from nonsmokers with respect to emotional status and other selected characteristics. From this sample, groups of cigarette smokers and nonsmokers were matched by age, sex, race, and social class. This comparison indicated that smokers differ from nonsmokers in their replies to questions concerning emotional status. Cigarette smokers marry more often, move more frequently, change jobs more often, are hospitalized more often, and participate in more sports than nonsmokers. Also, the parents of nonsmokers are more often foreign born than those of smokers. No differences were noted in birthplace, religion, education, present marital status, and proportion of lifetime spent in large cities. Possible interpretations of these results are discussed, emphasizing their bearing on the statistical association of cigarette smoking with lung cancer and other diseases. In the characteristics studied, the degree of difference between smokers and nonsmokers was not sufficient to account for the high degree of association between cigarette smoking and lung cancer and therefore could not influence the causal inference derived from this association, though it might be sufficient to explain the association of cigarette smoking with peptic ulcer and coronary artery disease. Further studies are needed to test these possibilities.—**J. Nat. Cancer Inst. 22: 259–282, 1959.**

One of the objections to interpreting the statistical association of cigarette smoking with lung cancer and other diseases as indicating a causal relationship is that the association may be a result of "self-selection" (*1*). Self-selection means that the unknown factors which cause an individual to smoke are also of etiological importance for lung cancer or other diseases. This type of reasoning is illustrated in an epidemiological investigation of frostbite, which was carried out in Korea during 1951–52. One aspect of this study consisted of a comparison of a

[1] Received for publication July 22, 1958.

[2] This investigation was aided in part by research grant CS-9408 from the National Cancer Institute, National Institutes of Health, Public Health Service.

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group of frostbite victims and of controls with respect to a number of characteristics, among which was smoking (*2*). This comparison indicated that there were more heavy cigarette smokers among the controls than among the frostbite victims (table 1). A causal interpretation of this association would be that heavy cigarette smoking had a protective influence against frostbite. The investigators did not interpret the data in such a manner, but thought that the heavy smoking habit was a reflection of intrinsic emotional and personality factors, and that these same factors were of etiological importance in frostbite. Thus, the negative statistical association of frostbite and smoking was considered to be a result of factors that selected individuals for both smoking and frostbite; at least it was so interpreted by those who carried out this study. Obviously, in an interpretation of statistical associations determined by epidemiological studies, it is important to evaluate the possible influence of this type of self-selection.

One approach to such an evaluation is the comparison of smokers and nonsmokers with respect to various characteristics. If smokers and nonsmokers are alike in a multitude of other characteristics, it would be difficult to accept a self-selection hypothesis. However, it should be emphasized that if differences are found between smokers and nonsmokers, it does not necessarily mean that the association between a particular disease and smoking is the result of selective factors. It is quite possible that the characteristics in which smokers and nonsmokers differ may be of no importance in terms of the association of smoking to the particular disease of interest. It would be necessary to determine, by further study, whether the characteristics distinguishing smokers from nonsmokers are independently related to the disease. If they are not so related, then "self-selection" cannot be a reasonable explanation for the association of smoking with disease. On the other hand, if they are related, "self-selection" may be a reasonable explanation. In line with this reasoning, information on the characteristics of a sample of smokers and nonsmokers in an urban population was collected during 1956. This is a report of some of the results of this survey that have a bearing on some of the issues discussed.

TABLE 1.—*Comparison of 691 victims of frostbite and 442 bunker-mate controls with respect to daily amount of smoking; Korea, 1951–52**†

Cigarettes per day	Frostbite victims		Controls	
	Number	Percent	Number	Percent
None	128	18. 5	83	18. 8
½ pack	211	30. 5	66	14. 9
1 pack	241	34. 9	179	40. 5
1½ packs	54	7. 8	54	12. 2
2 packs	46	6. 7	44	10. 0
Over 2 packs	11	1. 6	16	3. 6
Total	691	100. 0	442	100. 0

*From Schumann (*2*).
†Chi square = 42.877; degrees of freedom = 5; $P<0.0001$.

Method of Study

The information was obtained by interviewing all adult persons (18 years of age and over) in all households of a sample of addresses, and a sample of adults in lodging houses in Buffalo and Kenmore, New York. Residents of hospitals, convents, dormitories, etc., were not included in the sample, and therefore those interviewed represented the noninstitutionalized population. In both the regular addresses and lodging houses, the samples were selected so that they resulted in an over-all sampling fraction of 1 in 75.

A systematic sample of addresses was drawn from the Buffalo City Directory of 1956, supplemented by a list of new-building addresses obtained from permits issued by the Bureau of Buildings. To allow for omissions from the directory, the "half-open interval" method, described by Yates (*3*), was used; addresses thus obtained were added to the original lists. Independent, areal sampling studies indicated that very few addresses were missed by these methods. Special methods were used for sampling apartment houses, as described by Hansen *et al.* (*4*). The sampling of lodging houses was carried out from lists obtained from the Erie County Health Department where each lodging house is registered; a systematic sample of individuals was drawn from this list, with a sampling fraction of 1 in 75. After selection was completed, sample addresses were arranged into socioeconomic quartiles of the city population as determined from census-tract data. Addresses were assigned to interviewers so that each interviewer had a representation from all socioeconomic strata.

A total of 4,456 adults was interviewed. About 10 percent of the adults in the selected sample was not interviewed for various reasons. Some refused, others were too ill, and still others could not be contacted despite repeated attempts by the interviewers.

The information can be divided into 2 parts, depending on the method by which it was obtained. The following was obtained from each respondent by direct interview: age, sex, race, lifetime marital history, education, occupation, lifetime residence history, lifetime smoking history, history of hospitalizations, exposure to diagnostic and therapeutic X rays, attitudes toward smoking, and, from each woman, a complete menstrual and pregnancy history. An attempt was made to determine the emotional status of the respondents by means of a multiple-choice questionnaire, which was handed to the respondent to check the appropriate answer; this comprised a major portion of the second part of the interview. This check list included a question on religious preference and one on circumcision (the latter to be answered by males), which were considered too personal to be asked by the interviewers. Also included were several questions on participation in sports. In some instances, because of language or reading difficulties, these questions had to be asked by the interviewer, sometimes aided by an interpreter.

The questions on emotional status were obtained from a list used by Stouffer *et al.* in developing a Neuropsychiatric Screening Adjunct (*5*).

Stouffer and coworkers validated the items in the adjunct by questioning a group of "normals" and "neurotics"; the response for each item was reported. The 31 questions used in the present study were those in which there were differences in response between these "normal" and "neurotic" groups.

To compare cigarette smokers and nonsmokers, it was considered necessary that the 2 groups be alike with regard to age, sex, race, and social status, since these factors may be independently related to the characteristics of interest. Out of the total sample, a group of 903 present cigarette smokers was matched with a group of 903 nonsmokers, with respect to the four variables, age, sex, race, and social status. Nonsmokers are defined as those who had smoked less than 5 to 10 packs of cigarettes, less than 50 to 75 cigars, less than 3 to 5 packages of pipe tobacco, and had chewed less than 3 to 5 plugs of chewing tobacco during their lifetime. For matching by age, 5-year age groups were used rather than the exact age; for example, a smoker 23 years of age was matched with a nonsmoker of between 20 and 24.9 years of age. For social status, the matching was accomplished by using the social quartile of residence. These were determined as follows: The census tracts in Buffalo were ranked according to median monthly rental as determined by the 1950 census, and then assembled into quartiles so that approximately 25 percent of the city's population fell into each quartile. Kenmore, a Buffalo surburb, was considered as a separate unit for matching purposes. The median monthly rental was considered a valid index of relative socioeconomic status because of its high correlation with other indexes, such as family income, years of schooling completed, and occupation. Admittedly, this is not the most refined index of social status, but it was the most practical and appears adequate for the present purposes. The distribution of this matched group by age, sex, race, and social quartile is presented in tables 2 and 3. From these tables we note that a vast

TABLE 2.—*Distribution of matched group of cigarette smokers and nonsmokers, by age, race, and sex*

Age	White			Nonwhite			Both races		
	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes
18–19	14	18	32	0	5	5	14	23	37
20–24	33	68	101	1	6	7	34	74	108
25–29	24	70	94	3	14	17	27	84	111
30–34	17	74	91	3	12	15	20	86	106
35–39	15	90	105	2	11	13	17	101	118
40–44	19	60	79	1	6	7	20	66	86
45–49	20	72	92	2	5	7	22	77	99
50–54	15	58	73	2	2	4	17	60	77
55–59	18	40	58	2	2	4	20	42	62
60–64	16	25	41	2	0	2	18	25	43
65–69	14	12	26	1	0	1	15	12	27
70 and over	15	14	29	0	0	0	15	14	29
All ages	220	601	821	19	63	82	239	664	903

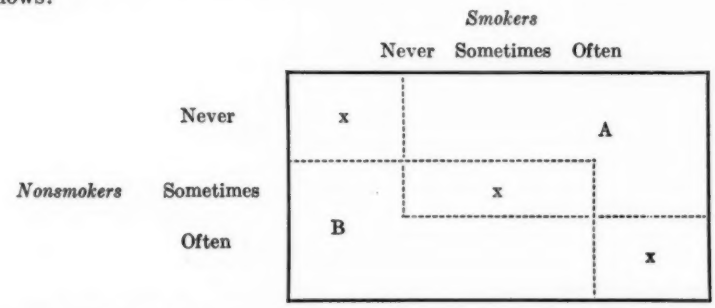
TABLE 3.—*Distribution of matched group of cigarette smokers and nonsmokers by race, sex, and social class*

Social quartile	White			Nonwhite			Both races		
	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes
Buffalo									
1 (highest)	78	165	243	0	1	1	78	166	244
2	40	155	195	2	12	14	42	167	209
3	52	145	197	9	25	34	61	170	231
4 (lowest)	41	116	157	8	25	33	49	141	190
Kenmore	9	20	29	0	0	0	9	20	29
Total	220	601	821	19	63	82	239	664	903

majority (about 75 percent) of the matched group are females. This sex distribution is different from that of the total sample because only about 50 percent of females smoke, whereas about 75 percent of males are cigarette smokers. Consequently, it was easier to obtain a matched group of females than of males.

It would be highly desirable to analyze all the available information from the sample and not limit it to matched pairs selected, but computational difficulties result if each of the responses is so analyzed. However, such an analysis is being initiated, utilizing an emotional score computed from the responses of all the items. The results will be reported when completed.

Comparison of smokers and nonsmokers is presented as the percent distribution of responses for each item in each group of questions with a qualitative response. In the statistical analysis of these results it was necessary to take into account the fact that we were dealing with matched groups. The responses of smokers and nonsmokers had to be cross-classified, and the chi-square test suggested by Cochran for matched groups was used to test statistical significance (*6*). For example, in a 3-category response, smokers and nonsmokers were cross-classified as follows:



The cells containing an x represent those responses in which smokers and nonsmokers agreed. The pooled cells designated by A represent smokers

who responded as having a characteristic more frequently than nonsmokers. Similarly, in regard to the nonsmokers, the cells are designated by B. Chi square was computed as $\frac{(A-B-1)^2}{(A+B)}$. The probability values for each of these tests are presented with each item in addition to the percent of matched pairs whose responses completely agree. For each of the responses on quantitative items, the mean differences, standard errors of mean differences, and the percent of agreements are presented; a t test for pairs was performed, and the probability levels of these tests are also presented for each item.

Results

Emotional Status

A comparison of the responses of cigarette smokers and nonsmokers to each question concerning emotional status is presented in table 4. We note that smokers were significantly different from nonsmokers in 16 out of the 31 items at probability levels of 0.02 or less. If 0.05 is used as the significance level, the 2 groups differed in 19 items. In all those questions in which the 2 groups were significantly different, the responses of smokers were excessive in the direction that might be considered "neurotic." No attempt will be made to define the term "neurotic." No doubt there are

TABLE 4.—*Response comparison of matched groups of cigarette smokers and nonsmokers in questionnaire on emotional status*

Question	Percent distribution of response of:		Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square *(1 degree of freedom)*	*P*
	Cigarette smokers	Nonsmokers			
1. Do you ever feel like smashing things for no good reason?					
Almost never	75. 5	84. 4	66. 4	21. 3	<. 001
Sometimes	22. 5	14. 9			
Very often	2. 0	0. 8			
2. How often does it make you sore to have people tell you what to do?					
Almost never	33. 4	42. 7	43. 7	20. 7	<. 001
Sometimes	56. 0	51. 6			
Very often	10. 5	5. 7			
3. Do your hands ever tremble enough to bother you?					
Never	80. 6	88. 4	72. 3	20. 33	<. 001
Sometimes	17. 1	10. 2			
Often	2. 3	1. 4			
4. Are you bothered with nervousness?					
Never	29. 7	40. 1	44. 2	19. 96	<. 001
Sometimes	56. 2	50. 3			
Often	14. 1	9. 6			

TABLE 4.—*Response comparison of matched groups of cigarette smokers and nonsmokers in questionnaire on emotional status*—Continued

Question	Percent distribution of response of:		Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square *(1 degree of freedom)*	*P*
	Cigarette smokers	Nonsmokers			
5. How often do people get on your nerves so that you want to do just the opposite of what they want you to do?					
Almost never	39.4	48.0	47.2	14.5	<.001
Sometimes	53.8	48.5			
Very often	6.8	3.5			
6. Do you find that you often have to tell people to mind their own business?					
Almost never	63.7	72.2	55.6	14.5	<.001
Sometimes	31.5	25.7			
Very often	4.8	2.0			
7. Are you ever troubled by your hands sweating so that they feel damp and clammy?					
Never	63.4	71.9	52.1	13.79	<.001
Sometimes	31.0	24.9			
Often	5.6	3.2			
8. Have you ever been bothered by shortness of breath when you were not exercising or working hard?					
Never	70.6	77.7	60.2	13.73	<.001
Sometimes	25.2	19.6			
Often	4.2	2.7			
9. Do you often say things you later wish you had not said?					
Almost never	21.5	28.1	52.9	12.3	<.001
Sometimes	70.8	66.7			
Very often	7.7	5.2			
10. Did you ever bite your fingernails when you were a child?					
Never	61.3	68.5	51.9	11.7	<.001
Sometimes	25.9	21.9			
Often	12.8	9.5			
11. Do you ever get so blue and discouraged that you wonder whether anything is worthwhile?					
Hardly ever	49.1	56.6	40.4	11.10	<.001
Not so often	18.0	18.6			
Sometimes	29.4	22.8			
Very often	3.5	2.0			

TABLE 4.—*Response comparison of matched groups of cigarette smokers and nonsmokers in questionnaire on emotional status*—Continued

Question	Percent distribution of response of:		Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*
	Cigarette smokers	Nonsmokers			
12. Are you ever worried and upset?					
Hardly ever	23. 9	28. 8	52. 5	9. 96	<. 01
Sometimes	66. 0	64. 4			
Often	10. 1	6. 8			
13. Have you ever been troubled by "cold sweats"?					
Never	76. 8	83. 1	66. 7	9. 04	<. 01
A few times	20. 3	15. 2			
Often	2. 9	1. 8			
14. Have you ever had spells of dizziness?					
Never	52. 9	60. 0	50. 0	7. 74	<. 01
A few times	42. 7	36. 8			
Many times	4. 4	3. 3			
15. Do you feel you get more than your share of bad luck?					
Almost never	53. 0	59. 4	46. 5	7. 02	<. 01
Sometimes	39. 7	36. 0			
Often	7. 3	4. 6			
16. Have you ever been bothered by your heart beating hard?					
Never	57. 8	64. 6	51. 3	5. 69	.02>*P*>.01
A few times	36. 6	30. 4			
Often	5. 6	5. 0			
17. In general, how would you say you feel most of the time; in good spirits or in low spirits?					
Good spirits most of time	66. 9	71. 8	58. 1	5. 36	.05>*P*>.02
Good spirits and low spirits equally	31. 3	26. 6			
Low spirits most of time	1. 8	1. 6			
18. Are you ever bothered by having nightmares?					
Never	68. 4	73. 0	58. 6	5. 25	.05>*P*>.02
A few times	29. 3	25. 2			
Many times	2. 3	1. 8			
19. Do you often have trouble in getting to sleep or staying asleep?					
Almost never	57. 4	62. 9			
Sometimes	32. 6	29. 2			
Often	10. 0	7. 9	49. 6	4. 07	.05>*P*>.02

TABLE 4.—*Response comparison of matched groups of cigarette smokers and nonsmokers in questionnaire on emotional status*—Continued

Question	Percent distribution of response of:		Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*
	Cigarette smokers	Nonsmokers			
20. How often do people hurt your feelings?					
Almost never	38. 9	41. 1	45. 5	3. 26	.10>*P*>.05
Sometimes	54. 3	54. 5			
Very often	6. 9	4. 4			
21. Have you ever had any fainting spells?					
Never	78. 6	81. 2	68. 1	2. 02	.20>*P*>.10
Sometimes	56. 2	50. 3			
Often	14. 1	9. 6			
22. Do you bite your fingernails now?					
Never	82. 1	84. 2	72. 5	1. 62	.20
Sometimes	11. 7	12. 0			
Often	6. 2	3. 8			
23. Have you ever been bothered by pressures in the head?					
Never	63. 8	67. 4	52. 6	1. 58	.30>*P*>.20
A few times	29. 0	27. 0			
Often	7. 2	5. 7			
24. Are you ever troubled with sick headaches?					
Never	53. 9	57. 5	47. 7	1. 04	.30
Sometimes	40. 6	36. 8			
Often	5. 5	5. 7			
25. How would you say people you know feel about you?					
Almost all like me	43. 1	45. 7	44. 6	0. 7	>.30
Most of them like me	54. 0	50. 6			
A few like me	2. 7	3. 7			
Almost none like me	0. 2	0. 0			
26. Do you worry very much about things that might happen to you?					
Not at all	25. 3	27. 6	41. 1	0. 6	>.30
Not very much	54. 7	52. 7			
Quite a bit	15. 4	15. 7			
A great deal	4. 6	4. 0			
27. When you were growing up, did you have any trouble with stuttering in your speech?					
Never	92. 3	93. 2	86. 0	0. 4	>.50
Sometimes	6. 3	5. 7			
Often	1. 3	1. 1			

TABLE 4.—*Response comparison of matched groups of cigarette smokers and nonsmokers in questionnaire on emotional status*—Continued

Question	Percent distribution of response of:		Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*
	Cigarette smokers	Nonsmokers			
28. How often are you bothered by having an upset stomach?					
Never	30.4	31.8	48.2	0.36	>.50
Not very often	60.7	61.1			
Pretty often	7.8	5.7			
Nearly all the time	1.1	1.4			
29. Do you have any particular physical or health problem?					
Yes	18.9	18.3	61.9	0.24	>.50
No	73.1	76.4			
Undecided	8.0	5.3			
30. As far as you know, were you a healthy child or a rather sickly one?					
Very healthy	71.1	70.6	59.8	0.3	>.50
Fairly healthy	25.9	24.9			
Rather sickly	3.0	4.5			
31. Do you usually like to be by yourself or to be with other people?					
With other people	80.4	80.5	65.7	0.00	.95
By myself	7.9	7.7			
Undecided	11.7	11.8			

many who would disagree with its use in the present context, but it is only being used to facilitate the presentation of results, and it should be viewed as merely indicating the types of response elicited by the specific questions. For our purposes it is sufficient to note that cigarette smokers as a group responded in a manner different from nonsmokers. Of interest is the distribution of items, according to the percent of responses, in which smokers and nonsmokers agreed. This is presented in table 5 where we note that, in a majority of the items, more than 50 percent of the matched groups' responses was in agreement.

Since the incidence of lung cancer is higher among males than females, it was of interest to make comparisons for males only. These results are presented in summary form in table 6, together with similar results of the total group to facilitate comparison. In this table, only the percent agreements, the chi-square values which were computed as described earlier, and the probability levels are shown. Unfortunately, the number of matched males is relatively small (239), which must be considered in evaluating this comparison. Of the 31 items, smokers differed from non-

TABLE 5.—*Distribution of items in questionnaire on emotional status according to percent responses in which cigarette smokers and nonsmokers agreed*

Percent responses in which cigarette smokers and nonsmokers agreed	Number of items in questionnaire
40–49	11
50–59	11
60–69	6
70–79	2
80–89	1
Total	31

smokers in 8, at probability levels of .05 or less. This decrease from the number in the total group should not be interpreted as indicating a decreased degree of association, since it may reflect the smaller numbers available for comparison. Similar comparisons were also made for a heavy-smoking category, defined as those who smoked 1 pack or more a day regularly; these are also presented in table 6. Here again, differences were statistically significant for 8 out of the 31 items, at probability levels of .05 or less. In general, for both males and heavy smokers, the items which differentiated smokers and nonsmokers were the same as for the total group, *i.e.*, the 16 significantly different items in the total group included the 8 items for each of these subgroups.

Another method of comparing smokers and nonsmokers was used for the individual items. For each item, smokers and nonsmokers were compared in terms of the direction of the response. For example, in table 4, item 1, smokers have a lower percentage of "never" responses than nonsmokers and a higher percent of "sometimes" and "very often." The direction of the response is toward what may be considered "neurotic." We can designate such a difference between smokers and nonsmokers as being "positive." If the nonsmokers' response for an item is in the "neurotic" direction, *i.e.*, more than that of the smokers', we can designate this difference as being "negative." If there is no difference between the 2 groups, we designate this as a "0." In some instances, there may be no consistent pattern, *i.e.*, smokers have less of a "never" response, more of a "sometime" response, and less of an "always" response; this type may be designated as "questionable." These designations have been applied to smoker-nonsmoker comparisons for each item and are presented in table 7. It is noteworthy that, for a vast majority of the items, smokers consistently responded in a "neurotic" direction. This was true for items in which no statistically significant differences in response were indicated by earlier analysis.

From these data it is apparent that the responses by cigarette smokers on the questions concerning emotional status were consistently more "neurotic" than those of nonsmokers. As mentioned earlier, these ques-

TABLE 6.—*Comparison of responses in questionnaire on emotional status for total matched group of cigarette smokers and nonsmokers, for male persons only, and for heavy smokers only*

Question	Total group			Males only			Heavy smokers		
	Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*	Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*	Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*
1. Do you ever feel like smashing things for no good reason?	66. 4	21. 3	$<.001$	71. 9	5. 97	$<.02$	63. 0	1. 96	$.2>P>.1$
2. How often does it make you sore to have people tell you what to do?	43. 7	20. 7	$<.001$	45. 0	3. 05	$.10>P>.05$	46. 0	10. 45	$<.001$
3. Do your hands ever tremble enough to bother you?	72. 3	20. 33	$<.001$	81. 8	4. 56	$<.05$	67. 6	5. 69	$<.02$
4. Are you bothered by nervousness?	44. 2	19. 96	$<.001$	45. 3	11. 19	$<.001$	40. 7	6. 94	$<.01$
5. How often do people get on your nerves so that you want to do just the opposite of what they want you to do?	47. 2	14. 5	$<.001$	48. 7	5. 98	$<.02$	47. 8	5. 01	$<.05$
6. Do you find that you often have to tell people to mind their own business?	55. 6	14. 5	$<.001$	48. 1	11. 04	$<.001$	54. 0	2. 64	$.2>P>.1$

7. Are you ever troubled by your hands sweating so that they feel damp and clammy?	52. 1	13. 79	<. 001	57. 1	0. 80	.5>*P*>.3	48. 6	1. 13	.3>*P*>.2
8. Have you ever been bothered by shortness of breath when you were not exercising or working hard?	60. 2	13. 73	<. 001	64. 4	10. 59	. 01	57. 9	6. 78	<. 01
9. Do you often say things you later wish you had not said?	52. 9	12. 3	<. 001	52. 9	1. 08	. 30	54. 4	1. 59	. 20
10. Did you ever bite your fingernails when you were a child?	51. 9	11. 7	<. 001	58. 1	0. 16	. 70	49. 6	0. 70	.5>*P*>.3
11. Do you ever get so blue and discouraged that you wonder whether anything is worthwhile?	40. 4	11. 10	<. 001	52. 7	4. 72	<. 05	36. 7	5. 01	<. 05
12. Are you ever worried and upset?	52. 5	9. 96	<. 01	48. 5	1. 60	. 20	50. 4	6. 49	<. 02
13. Have you ever been troubled by "cold sweats"?	66. 7	9. 04	<. 01	68. 6	0. 34	. 60	61. 4	2. 24	.2>*P*>.1
14. Have you ever had spells of dizziness?	50. 0	7. 74	<. 01	57. 1	0. 48	. 50	45. 0	1. 87	.2>*P*>.1
15. Do you feel you get more than your share of bad luck?	46. 5	7. 02	<. 01	51. 9	1. 27	.3>*P*>.2	43. 9	2. 88	.10>*P*>.05
16. Have you ever been bothered by your heart beating hard?	51. 3	5. 69	.02>*P*>.01	56. 1	0. 95	.5>*P*>.3	42. 9	1. 51	. 20

TABLE 6.—*Comparison of responses in questionnaire on emotional status for total matched group of cigarette smokers and nonsmokers, for male persons only, and for heavy smokers only*—Continued

Question	Total group			Males only			Heavy smokers		
	Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*	Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*	Percent of responses in which cigarette smokers and nonsmokers agreed	Chi square (*1 degree of freedom*)	*P*
17. In general, how would you say you feel most of the time, in good spirits or in low spirits?	58. 1	5. 36	.05>*P*>.02	67. 4	0. 32	.7>*P*>.5	61. 9	2. 72	.10
18. Are you ever bothered by having nightmares?	58. 6	5. 25	.05>*P*>.02	69. 2	1. 37	.3>*P*>.2	59. 0	2. 53	.2>*P*>.1
19. Do you often have trouble in getting to sleep or staying asleep?	49. 6	4. 07	.05>*P*>.02	59. 4	8. 08	<.01	47. 1	5. 96	<.02
20. How often do people hurt your feelings?	45. 5	3. 26	.10>*P*>.05	49. 0	1. 84	.20	46. 8	0. 12	.70
21. Have you ever had any fainting spells?	68. 1	2. 02	.20>*P*>.10	88. 9	0. 04	.85	74. 6	1. 03	.30
22. Do you bite your fingernails now?	72. 5	1. 62	.20	76. 8	0. 07	.80	73. 2	0. 97	.30
23. Have you ever been bothered by pressures or pains in the head?	52. 6	1. 58	.30>*P*>.20	66. 0	0. 20	.7>*P*>.5	52. 1	2. 15	.2>*P*>.1
24. Are you ever troubled with sick headaches?	47. 7	1. 04	>.30	60. 6	0. 39	.7>*P*>.5	47. 9	2. 68	.2>*P*>.1

25. How would you say people you know feel about you?	44.6	0.7	$>.30$	44.1	0.00	.98	44.9	3.80	$.10>P>.05$
26. Do you worry very much about things that might happen to you?	41.1	0.6	$>.30$	44.5	0.92	$.5>P>.3$	39.9	0.43	$.8>P>.7$
27. When you were growing up, did you have any trouble with stuttering in your speech?	86.0	0.4	$>.50$	79.9	0.02	.90	85.6	0.5	.80
28. How often are you bothered by having an upset stomach?	48.2	0.36	$>.50$	45.2	0.00	.98	45.7	0.33	$.7>P>.5$
29. Do you have any particular physical or health problem?	61.9	0.24	$>.50$	62.3	0.28	$.7>P>.5$	56.1	0.26	.60
30. As far as you know were you a healthy child or a rather sickly one?	59.8	0.3	$>.50$	61.9	0.18	.70	54.7	2.29	$.20>P>.110$
31. Do you usually like to be by yourself or to be with other people?	65.7	0.00	$>.95$	66.4	2.11	$.2>P>.1$	61.2	.02	.90

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TABLE 7.—*Comparison of cigarette smokers and nonsmokers with regard to direction of response on each item in questionnaire on emotional status for total matched group, males, and heavy smokers*

Item No.*	Direction of response			Item No.*	Direction of response		
	Total group	Males only	Heavy smokers		Total group	Males only	Heavy smokers
1	+†	+	+	17	+	+	?
2	+	+	+	18	+	+	+
3	+	+	+	19	+	+	+
4	+	+	+	20	+	+	+
5	+	+	+	21	+	−	+
6	+	+	+	22	+	?	+
7	+	+	+	23	+	+	+
8	+	+	+	24	?	+	+
9	+	+	+	25	?	?	+
10	+	−‡	?§	26	+	+	+
11	+	+	+	27	+	+	−
12	+	+	+	28	?	+	?
13	+	+	+	29	+	?	+
14	+	+	+	30	−	?	+
15	+	+	+	31	0‖	−	?
16	+	+	+				

*Item numbers refer to the numbered questions listed in table 4.
†+ = Smokers had more "neurotic" responses than nonsmokers.
‡− = Nonsmokers had more "neurotic" responses than smokers.
§? = No consistent pattern in smoker-nonsmoker comparison.
‖0 = No differences in response between smokers and nonsmokers.

tions were answered by the respondents themselves except, in a small number of instances, where assistance by the interviewer or other persons was necessary because of language difficulties. It is pertinent to inquire whether smokers and nonsmokers differed in this respect. In table 8, smokers and nonsmokers in this matched group are compared with respect to the means used in obtaining the responses. In a majority of instances (83 to 87 percent), respondents completed the questionnaire themselves, although 4 percent more nonsmokers required assistance than did smokers. Thus it is unlikely that the differences in response result from differences in methods of obtaining the information.

Participation in Sports

Detailed information on participation in various sports was obtained since it was thought that sports activity would reflect constitutional differences. For example, a high school athlete may not begin to smoke because of participation in athletics. Such an occurrence would suggest that nonsmokers, on the whole, might be constitutionally hardier than smokers.

A comparison of cigarette smokers and nonsmokers in sports participation is presented in table 9, which shows that, in 5 out of 8 sports, smokers participated to a greater extent than nonsmokers did, and these differences were statistically significant at a probability level of .01 or less. In one other sport, either tennis or badminton, the difference approached statistical significance with a probability level between .05 and .10. For a

TABLE 8.—*Comparison of cigarette smokers and nonsmokers in regard to persons who completed questionnaire on their emotional status*

Person completing questionnaire	Cigarette smokers		Nonsmokers	
	Number	Percent	Number	Percent
Respondent	769	87. 0	730	83. 0
Interviewer	98	11. 1	116	13. 2
Aided by other person	17	1. 9	34	3. 8
Total	884*	100. 0	880†	100. 0

*On 19 questionnaires this information was not stated.
†On 23 questionnaires this information was not stated.

TABLE 9.—*Comparison of cigarette smokers and nonsmokers with regard to sports participation during lifetime*

Participation in sports	Cigarette smokers	Non-smokers	Percent agreement	Chi square *(1 degree of freedom)*	*P*
Baseball:					
A lot	24. 1	18. 1			
A little	40. 1	36. 9	44. 9	24. 84	<. 001
Not at all	35. 9	45. 0			
Fishing:					
A lot	12. 2	8. 6			
A little	27. 9	23. 1	53. 1	17. 1	<. 001
Not at all	59. 8	68. 3			
Hunting:					
A lot	6. 6	3. 2			
A little	12. 5	7. 2	75. 5	29. 8	<. 001
Not at all	81. 0	89. 5			
Football or soccer:					
A lot	6. 1	7. 1			
A little	15. 8	11. 6	74. 3	1. 4	.3>*P*>. 2
Not at all	78. 1	81. 3			
Hockey:					
A lot	2. 2	2. 7			
A little	8. 2	6. 7	82. 9	0. 4	. 50
Not at all	89. 6	90. 6			
Basketball:					
A lot	18. 2	15. 2			
A little	34. 6	28. 9	48. 3	11. 2	<. 001
Not at all	47. 2	55. 9			
Tennis or badminton:					
A lot	9. 8	9. 0			
A little	31. 9	26. 3	53. 9	3. 69	.10>*P*>. 05
Not at all	58. 3	64. 8			
Golf:					
A lot	3. 0	3. 1			
A little	14. 9	9. 5	74. 7	7. 77	<. 01
Not at all	82. 1	87. 4			
Other sports:					
Yes	31. 3	27. 8	61. 9	4. 42	<. 05
No	68. 7	73. 2			

miscellaneous grouping of other nonspecified sports, smokers also had a greater frequency of participation, at a probability level of less than .05.

Respondents were also asked the number of sports they now participated in. Analysis of the matched group indicated that the average difference between smokers and nonsmokers was +.10 which was statistically significant at a probability level of .02 (*see* table 11).

In summary, these results indicate that cigarette smokers participate more frequently and in more sports than nonsmokers do, an observation that is different from what was expected. If the reasoning mentioned earlier is correct, these data would imply that cigarette smokers are hardier individuals than nonsmokers.

Other Characteristics

Smokers and nonsmokers were compared with regard to other characteristics, namely, religion, marital status, birthplace, birthplace of parents, education, lifetime histories of number of marriages, number of times employed, number of hospitalizations, number of different cities or towns lived in, number of X-ray or fluoroscopic diagnostic examinations received during a 12-month period preceding interview, and number of X-ray or radium treatments during a lifetime. The results of these comparisons are presented in tables 10 and 11.

No differences exist between smokers and nonsmokers with respect to religion, marital status, education, and respondent's birthplace. As to parents' birthplace, it is of interest that a significantly larger proportion of parents of nonsmokers were born outside the United States. The meaning of this difference is not clear.

Comparisons of nonsmokers and smokers with respect to the frequencies of marriage, etc. (table 11) indicate that in number of times employed, number of times married, and number of hospitalizations, cigarette smokers exceed nonsmokers. Cigarette smokers also move more frequently than nonsmokers do, though this difference barely approaches statistical significance. No differences were noted in diagnostic X-ray examinations or amount of X-ray or radium treament received during a lifetime.

The excesses noted for smokers in such variables as employment, marriages, etc. may be consistent with the results of the emotional-status questionnaire. A more "neurotic" group might tend to change jobs more often, move more often, etc. The excess number of hospitalizations is of interest and may also reflect "neuroticism." It would be of interest to determine the reasons for hospitalization, since these may reflect other reported associations between disease and cigarette smoking; such an analysis is in progress and will be reported later.

Lifetime Residence in Large Cities

In epidemiological studies of lung cancer, it has been found that lung cancer is more frequent in urban than in rural areas (*7*). This has led to the hypothesis that atmospheric pollution may be an etiological factor

TABLE 10.—*Comparison of cigarette smokers and nonsmokers with respect to selected characteristics*

Characteristic	Percent distribution of:		Percent of total group in which smokers and nonsmokers were similar	Chi square (*1 degree of freedom*)	*P*
	Cigarette smokers	Nonsmokers			
Religion:					
Catholic	58. 1	60. 0			
Protestant	36. 0	33. 9	54. 7	0. 90	.40>*P*>.30
Jewish	4. 4	3. 8			
Other	0. 9	1. 9			
None	0. 6	0. 4			
Present marital status:					
Married	71. 2	70. 8			
Widowed	7. 5	6. 2			
Divorced	2. 8	0. 6	60. 4	.00	>.90
Separated	4. 2	2. 3			
Never married	14. 0	19. 7			
Annulled	0. 3	0. 4			
Education (maximum level):					
None	1. 3	1. 8			
Some grade school	11. 3	13. 6			
Grade school completed	17. 7	16. 9	26. 2	0. 34	>.50
Some high school	30. 0	24. 3			
High school completed	21. 6	24. 6			
Attended college	18. 1	18. 8			
Parents' birthplace:					
Both born in United States	50. 8	45. 7			
One born in United States	15. 2	11. 6	49. 0	11. 58	<.001
Both born outside United States	33. 5	41. 8			
One or both unknown	0. 6	0. 9			
Respondent's birthplace:					
Buffalo	53. 9	54. 9			
Rest of United States	33. 6	30. 9			
Poland	1. 2	2. 6	47. 6	0. 2	>.60
Italy	1. 1	3. 7			
Other outside United States	10. 1	8. 7			

in lung cancer (*8*). However, since cigarette smoking is more frequent in urban than in rural areas, it has been necessary to adjust for cigarette smoking, in evaluating this "urban" factor. After such adjustment, the "urban" factor is still present (*7*). These adjustments have been limited to place of residence at a specific time (usually at the time of a

TABLE 11.—*Comparison of cigarette smokers and nonsmokers with respect to selected characteristics*

Characteristic	Total group			Heavy smokers only			Males only		
	Cigarette smokers minus nonsmokers: mean difference	t	P	Cigarette smokers minus nonsmokers: mean difference	t	P	Cigarette smokers minus nonsmokers: mean difference	t	P
1. Number of times employed	+.39	5.54	$<.001$	+.20	1.13	.25	+.14	0.67	.50
2. Number of times married	+.10	5.01	$<.001$	+.18	3.20	$<.01$	+.12	3.07	$<.01$
3. Number of hospitalizations	+.26	4.17	$<.001$	+.47	2.43	$<.02$	+.13	1.21	.20
4. Number of different residences	+.16	1.83	$.10>P>.05$	+.11	0.48	.65	+.13	0.84	.40
5. Number of X-ray or fluoroscopic examinations during 12 months preceding interview	−.002	0.03	$>.95$	+.21	1.21	.25	+.14	0.92	.35
6. Number of X-ray or radium treatments during lifetime	−.20	0.83	.40	+.15	1.38	$.25>P>.10$	+.10	1.66	$.10>P>.05$
7. Number of sports	+.10	2.33	.02	+.02	0.17	.85	−.01	0.12	.90

census) and have not taken into account lifetime residential histories. If nonsmokers who now reside in urban areas had spent most of their lives in rural areas, and if smokers had spent most of their lives in urban areas, it is possible that the nonsmoker-smoker differences in lung cancer mortality may reflect, at least partially, lifetime differences in exposure to the "urban factor." The present data permitted a study of this possibility, since lifetime residential histories were obtained from the respondents. In 655 of the matched pairs it was possible to categorize the respondents as to the percentage of their lives spent in cities of varying size. Nonsmokers spent 1.5 percent more of their lives in cities with a population of at least 100,000 than cigarette smokers did; this difference was not statistically significant.

Discussion

A comparison of cigarette smokers and nonsmokers has indicated that smokers differ from nonsmokers in their replies to a questionnaire concerning emotional status. Smokers marry more often, move more frequently, change jobs more often, are hospitalized more often, and participate in more sports than the nonsmokers do. In addition, the parents of nonsmokers are more often foreign born than those of smokers. These differences with respect to emotional factors agree in a general fashion with other reports of similar studies carried out on select population groups (*9*). Similarities between smokers and nonsmokers were also observed. No differences were noted with respect to birthplace, religion, education, present marital status, and proportion of lifetime spent in large cities.

TABLE 12.—*Distribution of items by percent agreement of response*

Percent distribution of those who agreed on both interviews	Number of items
90–99	2
80–89	10
70–79	8
60–69	8
50–59	3
Total	31

In evaluating the responses to items in the questionnaire on emotional status, it is of considerable interest to have some idea of their reliability. An attempt was made in regard to this by interviewing a 10 percent systematic sample of that half of the respondents who were interviewed first; a total of 242 reinterviews were completed. A detailed report of this analysis of reliability is being prepared for publication elsewhere. To summarize briefly the results, in table 12 we have presented the distribution of items according to the percent agreement of response on both interviews. We note that in 12 items 80 percent or more of the

respondents agreed on both interviews. In general, the reliability of response to these items may be considered as being moderate; in fact, it is better than what some investigators would expect. Unless cigarette smokers and nonsmokers differ in reliability, and our data are not sufficient to evaluate this possibility, the existence of a moderate rather than an excellent degree of reliability would tend to diminish the degree of differences observed between smokers and nonsmokers. Of course, if they do differ in reliability, it is impossible to state the degree to which this difference would have affected our observations.

The results of the present study may be interpreted in various ways. One possible interpretation is that cigarette smoking may be a cause of the traits observed among smokers; this may not appear likely, but it is possible. The only way to test such a possibility would be to obtain a sample of 12- to 13-year-old school-age children who have not yet started to smoke, subject them to a battery of psychological tests, and then observe them for a number of years to determine whether children with "neurotic" traits tend to smoke more or less or equally as often as those without such traits. These children should be observed to determine what changes do occur in emotional status after they have started to smoke, and comparisons should be made with those who did not begin smoking.

Another interpretation of these data would be that "neurotic" traits lead to the initiation of the smoking habit. Perhaps this is a more likely possibility, which would indicate that smokers are a self-selected group, as discussed earlier. The proposed study discussed in the preceding paragraph would also provide a direct test of this hypothesis.

A third possibility is that smoking and "neuroticism" both result from common underlying factors. Perhaps certain social factors are interrelated with smoking habits, lung cancer, and emotional status. This is a form of the self-selection hypothesis, one step removed from the lung cancer-smoking association. However, the explanation is unlikely and difficult to test at our present level of knowledge.

Our major interest in carrying out this study was to obtain information about the self-selection hypothesis as a possible explanation of the association of lung cancer with cigarette smoking. This question would be answered more completely with data from the suggested study of school children. Since we have indicated that the most likely explanation for the observed differences is self-selection, it is of interest to assess these results in terms of the lung cancer-smoking association.

A self-selection explanation for the lung cancer-smoking relationship implies that emotional factors, within the context of the specific questions used in this study, are of etiological importance in lung cancer. In other words, cigarette smokers tend to develop more lung cancer than nonsmokers because they are more "neurotic." There is no evidence available with which such a possibility can be directly evaluated. For a direct test of this hypothesis, it would be necessary to select groups of smokers and nonsmokers among lung-cancer patients and groups of smokers and nonsmokers among controls, and subject them to a battery of psychological

tests to determine whether or not emotional factors may be considered as being independently related to lung cancer. In view of the results of the present study, such an investigation would appear profitable.

The self-selection hypothesis can also be evaluated in terms of our knowledge of the epidemiology of lung cancer. First, the degree of association between emotional factors and cigarette smoking, as determined in the present study, does not appear to be sufficiently high to be a likely explanation of the marked association between cigarette smoking and lung cancer. To obtain some idea of the degree of association between emotional response and cigarette smoking, we thought it would be of interest to compute the risk of cigarette smoking for a "neurotic" response relative to that of a "nonneurotic" response, with a method suggested by Cornfield (*10*). Since the responses were either in 3 or 4 categories, computations were first carried out under the assumption that the most "neurotic" category was a "neurotic" response and the remainder "nonneurotic." Similar computations were then made under the assumption that the two most "neurotic" categories were a "neurotic" response and the remainder was "nonneurotic." The highest relative risk obtained was 2.6. Obviously this degree of association is not sufficient to account for the tenfold excess of lung-cancer mortality for cigarette smokers as compared to that of nonsmokers (*11*). Admittedly, our method of measuring emotional status is rather crude and perhaps more refined methods might increase the degree of difference, but this cannot be assumed on the basis of the present data.

Another phenomenon would have to be explained to make the self-selection hypothesis tenable, namely, the marked increase in lung-cancer mortality during the past 2 to 3 decades (*12*). It would be necessary to assume that the frequency of "neurotics" in the population has also increased during the same period. Although definitive evidence on this point is actually not available, such a marked change probably has not occurred. On the basis of present evidence, the fact that cigarette smokers are more "neurotic" than nonsmokers does not detract from the hypothesis that cigarette smoking is an etiological factor in lung cancer.

It is possible that the "neurotic" traits of cigarette smokers may be an explanation, in whole or in part, of the association between cigarette smoking and other diseases, such as coronary artery disease and peptic ulcer, each of which may be related in some manner to psychological factors. For instance, the association between these diseases and cigarette smoking is not as strong as the association between cigarette smoking and lung cancer. Also, the increase of coronary artery disease mortality has not been as great as that of lung cancer during the past 2 to 3 decades (*13*). With respect to peptic ulcer, it is thought that, in Britain, the incidence of gastric ulcer has diminished, whereas that of duodenal ulcer has increased, although in the latter the statistical association with cigarette smoking is less (*14*). Thus, self-selection may be a reasonable explanation for the association of peptic ulcer and coronary disease with cigarette smoking. Studies of the relationship of emotional factors to

coronary disease and peptic ulcer among nonsmokers should be carried out to shed further light on such possibilities.

Further analysis of data obtained in the present survey is in progress and will be reported at a later date. It would be of interest to determine if there is a relationship between other forms of tobacco use and emotional factors. Unfortunately, matched comparisons, similar to the one used in this report, were not possible because of the small numbers, and the data will have to be analyzed by other more complex methods. Also the relationship between factors used in matching, such as social class, age, etc., and smoking habits and emotional factors is of interest and is being analyzed.

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Host-Virus Relationships in Rous Sarcoma Tissues *In Vitro*. II. Growth of Extraneous Viruses in Rous Sarcoma and Adult Chick Cells *In Vitro* [1, 2]

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Summary

The susceptibility of Rous sarcoma cells and adult chick fibroblastic cells to infection with several viruses, when cultivated in monolayers *in vitro*, was investigated. There appears to be no difference between the two types of cells in respect to susceptibility to infection. Newcastle disease virus, the NWS strain of influenza A virus, Western equine encephalitis, psittacosis, vaccinia, and herpes simplex viruses multiplied in both types of tissue, while PR8 and LEE strains of influenza virus did not multiply in either. Multiplication was always accompanied by a cytopathogenic effect, and with destruction of tumor cells there was a loss of tumor-inciting ability in the culture fluid, except for vaccinia virus. This latter agent showed cellular destruction, but there were always viable cells remaining at the end of the experiment. The Rous sarcoma virus does not appear to act as an effective interfering agent for these other viruses. In fact, these experiments indicate that dual infection of the transformed tumor cell by the tumor virus and by one of a variety of extraneous viruses is probably the rule rather than the exception.—**J. Nat. Inst. Cancer 22: 283–295, 1959.**

Preliminary studies from this laboratory (*1*) indicated that Rous sarcoma cells cultivated *in vitro* were able to support the multiplication of at least one extraneous virus—Newcastle disease virus. In addition, there appeared to be no difference between normal adult chick tissue and tumor tissue in respect to viral susceptibility. This paper is the result of further attempts to assess the ability of adult chick cells and virus-induced Rous sarcoma cells to support the multiplication of a number of viruses whose growth characteristics are well known. These studies were undertaken to elucidate further the differences and/or similarities between "normal" and tumor cells as well as to establish the occurrence of viral interference or dual infection in the tumor cells.

The results indicate that Newcastle disease virus, the NWS strain of influenza A virus, vaccinia virus, Western equine encephalitis virus,

[1] Received for publication July 23, 1958.

[2] This investigation was supported by research grant C-2223 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

herpes simplex virus, and the 6BC strain of psittacosis virus multiplied in and exerted a cytopathogenic effect on both adult chick cells and Rous sarcoma cells *in vitro*. Influenza A virus strain PR8 and B strain LEE did not multiply in either type of cell.

Materials and Methods

Viruses.—The Rous sarcoma virus preparations for the induction of tumors were the partially purified lots CT–581 and CT–669 (*2*), kindly supplied by Dr. W. R. Bryan. The virus was stored in glass ampoules in a dry-ice chest at −70° C. The Newcastle disease virus strain B and the influenza virus strains PR8, NWS, and LEE had been passed repeatedly in the allantoic sac of 9-day embryonated eggs. Stock virus was prepared by harvesting allantoic fluids 48 hours after inoculation, centrifuging, and removing the supernatant for storage in ampoules at −70° C. The NWS strain of influenza was lethal to mice and multiplied to high titer in their brains. The Western equine encephalitis virus had been passed repeatedly in the yolk sac of 7-day embryonated eggs. Stock suspensions were prepared by harvesting infected embryos 18 to 36 hours after yolk-sac inoculation, grinding with mortar and pestle, diluting to a 10 percent suspension by weight in broth, centrifuging, and storing at −70° C. The 6BC strain of psittacosis virus had been passed repeatedly in 7-day embryonated eggs by the yolk-sac route. A uniform source of the agent was obtained as described previously (*3*). The vaccinia virus was passed repeatedly on the chorio-allantoic membrane of 12-days eggs. Stock virus was prepared by harvesting chorio-allantoic membranes 72 hours after inoculation, grinding with mortar and pestle, diluting in broth to a 10 percent suspension by weight, centrifuging, and storing the supernatant at −70° C. The herpes simplex virus had been passed repeatedly in yolk sacs of 7-day embryonated eggs. Stock suspensions of virus were prepared by harvesting yolk sacs 3 to 5 days after infection, homogenizing, diluting in broth to a 10 percent suspension by weight, centrifuging, and storing the supernatant at −70° C.

Virus titration.—The presence of Rous sarcoma virus in the cultures was indicated by the ability of a 1:2 dilution of culture fluids or a suspension of ground tissues to produce palpable tumors within 15 days after the subcutaneous injection of 0.25 ml. into the wing webs of 6 or more White Leghorn chicks, 12 to 21 days old.

Influenza strains PR8, NWS, and LEE, and the Newcastle disease virus were titered by making tenfold serial dilutions of the experimental fluids in chilled infusion broth and inoculating four 9-day embryonated eggs, by the allantoic route, with 0.25 ml. of fluid for each dilution. After the eggs were incubated at 35 to 36° C. for 48 hours, 0.5 ml. of allantoic fluid was removed from each egg and checked for the presence of virus by testing for hemagglutinins. The 50 percent infectivity end point was calculated according to the method of Reed and Muench (*4*).

Vaccinia, herpes simplex, and Western equine encephalitis viruses were titered by making tenfold serial dilutions of the experimental fluids in

chilled infusion broth, and inoculating four 7-day embryonated eggs by the yolk-sac route, with 0.25 ml. of fluid for each dilution. The eggs were incubated at 35 to 36° C. Embryos dead after 8 hours' incubation, in the case of Western equine encephalitis, and after 48 hours' incubation, in the case of vaccinia and herpes simplex, were discarded. Deaths were recorded through the 10th day after inoculation and the 50 percent lethal end point was calculated as described.

The psittacosis virus was titered by the single dilution method of Golub (*5*).

Culture media.—The following culture media were employed: A: chicken serum, 20 percent; gelatin, 0.5 percent; yeast extract, 0.1 percent; lactalbumin hydrolysate, 0.5 percent; Earle's balanced salt solution, 78.9 percent; B: chicken serum ultrafiltrate, 25 percent; gelatin, 0.5 percent; yeast extract, 0.1 percent; lactalbumin hydrolysate, 0.5 percent; Earle's balanced salt solution, 73.9 percent; C: chicken serum, 20 percent; Puck's medium C_1 (*6*), 80 percent; D: chicken serum ultrafiltrate, 25 percent; Puck's medium C_1, 75 percent. Media were buffered with 4 percent sodium bicarbonate and contained 50 γ per ml. of soybean trypsin inhibitor, 100 units per ml. of penicillin, and 50 γ per ml. of streptomycin, except for the psittacosis culture medium which contained no penicillin.

Medium A was used for the vaccinia and Western equine encephalitis Rous cell experiments, medium B for all other Rous cell experiments, medium C for the vaccinia-adult chick cell experiments, and medium D for all other adult chick cell experiments.

The chicken serum and plasma were always tested by injecting them, in various dilutions with Rous sarcoma virus, into chick wing webs to insure the absence of inhibitors to the tumor virus.

Tissues.—The Rous sarcoma tissue was obtained from tumors produced in the wing web by the injection of 0.25 ml. of a 1:30 dilution of Rous virus into chicks 12 to 20 days old. Malignant tissue was removed 8 to 10 days after inoculation from rapidly developing tumors showing no necrosis. Care was taken to avoid the inclusion of the normal tissues surrounding the tumor.

The adult chick tissue was obtained by removing the muscle and connective tissue adjacent to the wing web of chicks 18 to 25 days old.

Tissue-culture technique.—The Rous sarcoma tissue was minced, washed twice with balanced salt solution, and subjected to 3 separate treatments for 20 minutes with 0.25 percent trypsin (Difco 1:250) at 37° C. The 3 supernatants were pooled, put through a strainer, and centrifuged at 700 rpm for 5 minutes. The supernatant was discarded and the cells were diluted in medium to a concentration of 1.2 million cells per ml. One and five-tenths ml. of this cell suspension was introduced into each of a series of T-9 flasks (Konte Glass Co., Vineland, N. J.), the bottoms of which were coated with clotted rooster-plasma. The cultures were incubated at 37° C.

The adult chick tissue was minced, washed twice in balanced salt solution, and suspended in a small quantity of chick embryo extract

(50 percent balanced salt solution). Five-tenths ml. of this suspension was introduced into each of a series of T-9 flasks containing 0.5 ml. of liquid rooster-plasma. After 5 minutes the suspension had clotted and 1.5 ml. of medium was added to each flask. The flasks were incubated at 37° C. with daily changes of medium. On the 4th or 5th day the flasks were trypsinized with 0.25 percent trypsin for 30 minutes. The supernatant fluids were removed, strained, and centrifuged at 700 rpm for 5 minutes. The supernatant was discarded and the cells were diluted to a concentration of 200,000 cells per ml. One and five-tenths ml. of this cell suspension was introduced into each of a series of T-9 flasks, the bottoms of which were coated with clotted rooster-plasma. The cultures were incubated at 37° C.

At the time of virus inoculation, the number of cells in the tumor were comparable to those in adult chick monolayers, as determined by microscopic examination.

Twenty-four hours after initiation of the cultures, both the Rous sarcoma and adult chick cell monolayers were treated identically. The fluids were removed and the cultures washed twice with balanced salt solution. At least 4 flasks were inoculated with each virus diluted in the culture medium. After 4 hours' (2 hours in the case of Western equine encephalitis) incubation at 37° C., the fluids were removed, pooled, and centrifuged at 2000 rpm for 10 minutes. The supernatant fluid was removed and quick-frozen for subsequent virus assay. The cultures were washed twice with balanced salt solution, and fresh medium was added to all cultures. Thereafter, the fluids were removed at daily intervals and treated for virus titration as above. The monolayers were always washed with 1.5 ml. of balanced salt solution before the addition of fresh medium. During the course of some of the experiments with Western equine encephalitis and vaccinia, the clot and cells from single flasks were removed on various days, homogenized in 1.5 ml. of balanced salt solution with mortar and pestle, centrifuged, and the supernatant was frozen and stored for virus titration. In the adult chick cells this daily titering procedure was repeated for 10 days. With the Rous cells, the procedure was repeated for at least 10 days or until no extraneous virus was detectable in the culture fluids, except for vaccinia virus which was followed for periods up to 16 days.

Controls.—In each experiment, at least 2 cultures were treated in the same manner as described, except that fresh medium was substituted for the original virus inoculum. These cultures are subsequently referred to as tissue controls.

Virus controls to determine heat inactivation of the extraneous virus were prepared by incubating at 37° C. a series of 1.5 ml. aliquots of the original virus inoculum for each experiment. At appropriate intervals an aliquot was frozen for subsequent virus titration.

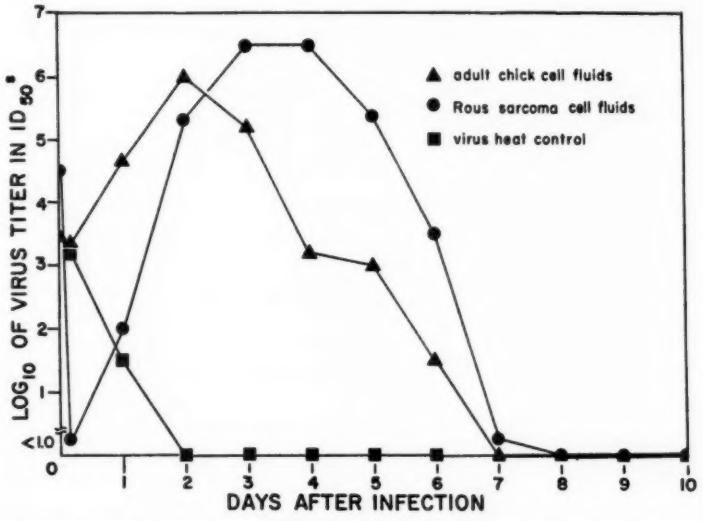
Individual experiments with each type of virus and cell system were repeated at least twice.

Results

The Rous sarcoma cells readily formed a monolayer on the plasma clot after 18 to 24 hours' incubation at 37° C. The cells were predominantly spindle-shaped, though there was a small proportion of round cells present, and nearly all the cells were vacuolated. During each experiment the cells increased in size, but there was no attempt made to determine definitely the extent of multiplication, if any. The Rous cells liberated large amounts of acid as reflected in the extremely rapid drop in culture fluid *p*H. This phenomenon has also been noted by Lo *et al.* (7).

The adult chick cells also readily formed monolayers after 18 to 24 hours' incubation at 37° C. All the cells were spindle-shaped and showed clear cytoplasm, particularly when medium D was used. In the latter instance it was necessary to stain representative cultures with neutral red to see clearly the cells in the monolayers. In contrast to the tumor cells, the culture media employed with the adult cultures showed very little, if any, gross *p*H change from day to day. Cells of this type multiply readily on glass, but plasma was used to make cultural conditions comparable to those of the Rous cells.

Text-figure 1 represents a typical experiment demonstrating the multiplication of Newcastle disease virus strain B in the tumor or adult cells. While the heat controls were no longer infective at 2 days, the titer of the virus in the Rous tumor fluids reached a maximum between the 2d and 4th days after infection. The virus titers decreased rapidly after the 4th day until there was no extraneous virus activity in the culture fluids



TEXT-FIGURE 1.—Growth curve of Newcastle disease virus in Rous sarcoma cells and in adult chick cells *in vitro*.

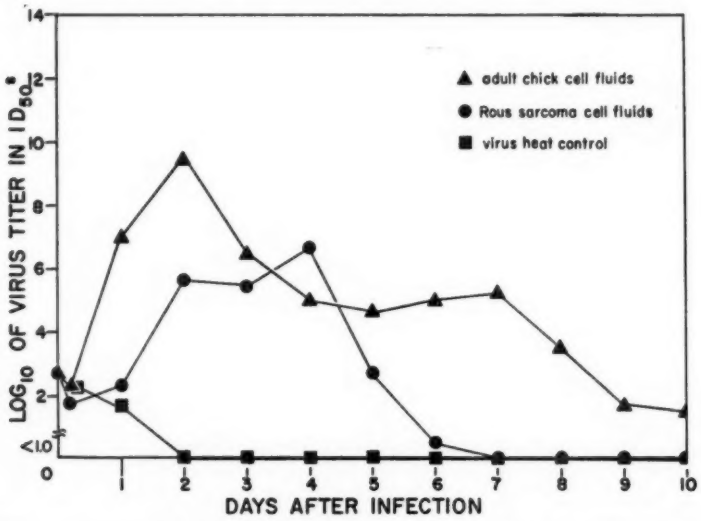
after 7 days. Microscopic observation of the tumor monolayers during the course of each experiment showed a progressive degeneration of cells in the infected flasks as opposed to cells in the control flasks, which were not exposed to infecting virus. There was little or no *p*H drop in the infected cultures after the 3d day. The control cultures continued to show a rapid *p*H drop throughout the course of the experiments.

Text-figure 1 also shows that Newcastle disease virus strain B multiplied in the adult chick tissue cells with a growth curve similar to that exhibited in the tumor cells. Periodic staining of representative monolayers with neutral red showed that the multiplication of the virus in adult chick cells was accompanied by such a cytopathogenic effect as occurred with the Rous tumor cells.

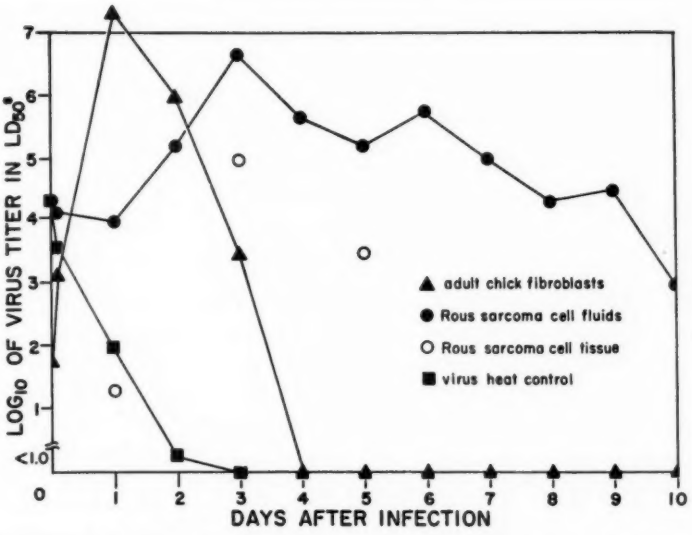
The experiments with the NWS strain of influenza A virus are summarized in text-figure 2. This virus showed multiplication in Rous cells which was comparable to that outlined with Newcastle disease virus. The NWS strain also showed a definite cytopathogenic effect on the tumor cells, which was also reflected in the loss of the characteristic change to an acid *p*H in the cultures.

Text-figure 2 also shows that the NWS strain multiplied in adult chick cells and maintained a significant titer in the infected fluids up to 10 days after the initial viral infection. While the NWS strain showed a definite cytopathogenic effect on the tumor cells by the 4th day, the cytopathogenic effect on the adult cells did not become marked until after the 6th day.

As can be seen in text-figure 3, Western equine encephalitis virus showed a rapid increase in titer in the tumor cells to a maximum between the 3d



TEXT-FIGURE 2.—Growth curve of NWS strain of influenza A virus in Rous sarcoma cells and in adult chick cells *in vitro*.



TEXT-FIGURE 3.—Growth curve of Western equine encephalitis virus in Rous sarcoma cells and in adult chick cells *in vitro*.

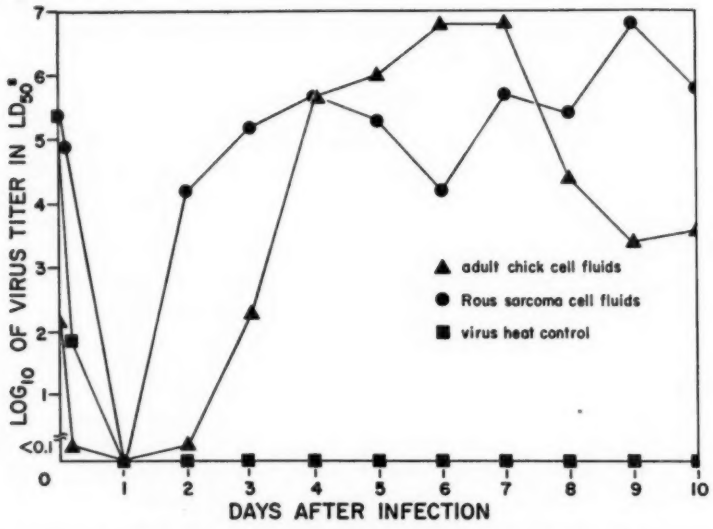
and 6th days, followed by a slow decrease in viral activity. The cytopathogenic effect of Western equine encephalitis on the infected monolayers of malignant cells began to become evident on the 3d day after infection and progressed to destruction of nearly all the cells. This cytopathogenicity was reflected by progressively smaller *p*H changes in the infected flasks from the 3d day to the completion of the experiments.

The adult chick cells showed a very rapid multiplication of Western equine encephalitis virus to a high titer on the 1st day, followed by a rapid decrease in titer to a complete absence of infective virus by the 4th or 5th day of the experiment. By this time the cells had been almost completely destroyed by the cytopathogenic effect of the virus.

Text-figure 4 indicates that psittacosis-infected tumor-cell fluid showed an initial drop in infectivity as virus was absorbed, followed by a steady increase in titer to significant levels for an extended period, which in some experiments was as long as 16 days. Failure of the infected flasks to show the characteristic rapid *p*H drop first became evident on about the 5th day after infection. Microscopic evidence of cytopathogenicity became evident on the same day and became increasingly apparent as the experiments progressed.

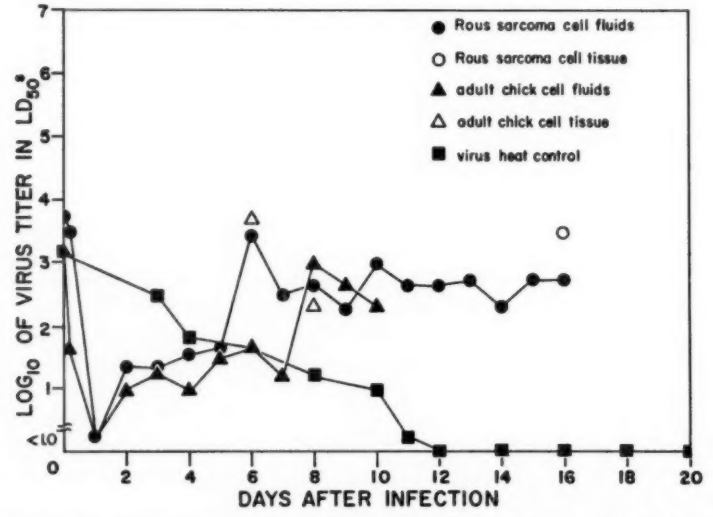
Psittacosis virus multiplied in adult chick cells with a growth curve similar to that obtained with the tumor-cell monolayers. The cytopathogenic effect of the psittacosis virus also became evident on the 5th or 6th day after infection.

As shown in text-figure 5, the growth curve of vaccinia virus in both the Rous and adult chick cells was not like that of the 4 viruses dis-



TEXT-FIGURE 4.—Growth curve of 6BC strain of psittacosis virus in Rous sarcoma cells and in adult chick cells *in vitro*.

cussed earlier. In the tumor cells the vaccinia virus titers showed a marked drop on the 1st day after infection, followed by a slow increase in titer to a significant level which was maintained for an extended period of days. Fluid viral titers between 10^2 and 10^3 were maintained



TEXT-FIGURE 5.—Growth curve of vaccinia virus in Rous sarcoma cells and in adult chick cells *in vitro*.

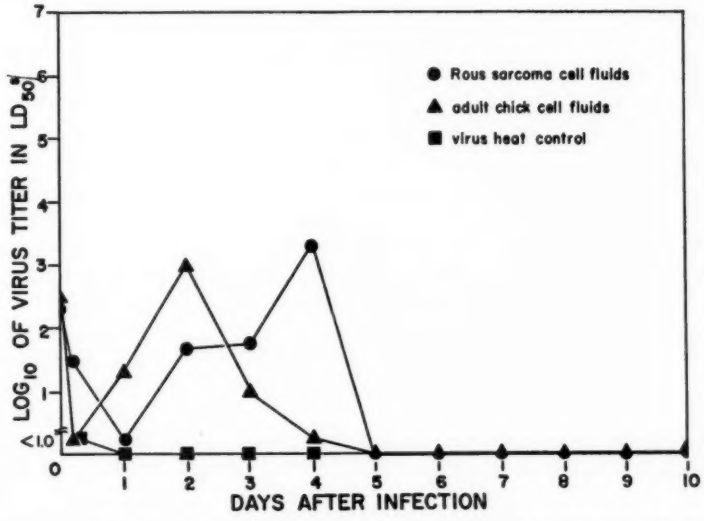
for as long as 16 days after initial viral infection. It should be noted that the tissue showed virus titers as high as $10^{8.5}$ on the 16th day in certain experiments.

A cytopathogenic effect on the Rous cells by the vaccinia virus became evident with the appearance of plaques on the 6th or 7th day after viral infection. These plaques developed into increasingly extensive zones of degenerated cells, but there were still considerable viable tumor cells remaining as long as 16 days after infection. This is in marked contrast to the results with other viruses multiplying in the Rous cells.

Text-figure 5 also shows that vaccinia virus multiplied slowly in adult chick cells after an initial rapid drop in fluid titer. The growth curve in this system was very similar to that in the tumor cells. Plaques reflecting the cytopathogenic effect of vaccinia virus on adult chick cells were also evident 6 days after initial infection.

As shown in text-figure 6, herpes simplex virus showed an initial drop in titer after infection, followed by an increase to a significant titer on the 3d or 4th day after infection and then a steady decrease to an absence of virus in the fluid by the 8th day. The multiplication of herpes simplex in the Rous cells was accompanied by a cytopathogenic effect that was readily evident by the 4th day. Herpes simplex also appeared to multiply in the adult chick tissue but to a less striking degree than most of the other viruses. This low grade multiplication was accompanied by a cytopathogenic effect as in the tumor cells. This effect was evident on the 4th or 5th day after infection.

Influenza virus strains PR8 and LEE failed to multiply in Rous sarcoma cells or in adult chick cells *in vitro*. The tumor cells continued to show a



TEXT-FIGURE 6.—Growth curve of herpes simplex virus in Rous sarcoma cells and in adult chick cells *in vitro*.

marked *p*H drop from day to day. There was no evidence of a cytopathogenic effect on either type of cell by these viruses.

In all experiments the fluids from control cells or homogenized control cells showed no effect when injected into eggs by either the allantoic or yolk-sac routes. Harvests of allantoic fluids from these eggs showed no evidence of hemagglutination. Allantoic or yolk-sac injection of high concentrations of purified Rous sarcoma virus also showed no effect on egg viability or production of hemagglutinins in allantoic fluids.

The injection into wing webs of fluids or viral-destroyed cell material from tumor cultures, which no longer showed any evidence of extraneous virus activity, never produced tumors within 15 days. However, fluids from vaccinia-infected flasks produced tumors, since the extraneous virus was still multiplying slowly at the termination of the experiments, and many viable tumor cells were evident. Fluids from control flasks and flasks containing nonmultiplying extraneous viruses always produced tumors in wing webs within 15 days.

Discussion

The results indicate that many types of extraneous viruses are able to multiply in Rous sarcoma cells *in vitro*. All 6 of the viruses tested have been shown to multiply in some type of tumor tissue *in vivo* or *in vitro* (*8*). However, these viruses exhibit varied properties in respect to multiplication and cytopathogenic effect in different virus-cell systems.

The evidence at present indicates that at any given time only a fraction of the Rous sarcoma cells liberates virus, but during a period covering approximately one generation time of the cells, all the tumor cells produce some virus (*9*). This evidence indicates that all the Rous sarcoma cells are infected with the tumor virus, even though they may not be liberating infective virus at a particular moment. The fact that Newcastle disease virus, NWS strain, Western equine encephalitis, and psittacosis viruses multiplied in the malignant cell cultures to titers greater than 10^6 indicates that at least a portion of the cells were dually infected by the tumor virus and the extraneous agent. It is, of course, impossible to tell from these experiments whether a Rous sarcoma cell is able to liberate the tumor virus and the extraneous virus at the same time.

It is noteworthy that the NWS strain of influenza virus multiplied in both the adult and the tumor tissue, while the PR8 and LEE strains did not multiply in either type of tissue. There is no evidence that the failure of the latter strains of influenza virus to multiply in the malignant cells was due to an interference phenomenon by the Rous virus. In addition to the fact that these agents did not multiply in the adult chick fibroblastic tissue, Stulberg and Schapira have reported that PR8 influenza virus did not multiply in fibroblasts derived from chick-embryo skeletal muscle (*10*). Thus the failure of these viruses to multiply in the Rous sarcoma cells appears to be due to tissue tropism rather than to an interference phenomenon.

The adult chick fibroblastic cells showed the same susceptibility to viral infection as the Rous sarcoma cells. A comparison of the viral

growth curves, together with appearance and extent of the cytopathogenic effect, both in these experiments as well as in unpublished data, indicates that there are no consistent differences between the two types of tissues in regard to their susceptibility to infection with the extraneous viruses used. It appears that the transformed tumor cells retain certain basic biologic characteristics common to adult cells despite the presence of the tumor virus. This conclusion is also consistent with that of Smith and Morgan (*1*), who found that in Maitland cultures Newcastle disease virus multiplied in both adult chick and Rous sarcoma tissues, and that vaccinia multiplied in adult chick tissues and possibly slowly in Rous sarcoma tissues, while the NWS strain of influenza virus and the 6BC strain of psittacosis virus did not multiply in either type of tissue. Dissimilarities in results of earlier work with individual viruses (*1*) and of experiments reported here probably can be traced to differences in the tissue-culture methods employed, since there is greater opportunity for virus infection of and replication in a monolayer of cells than in the fragments of tissue in fluid medium of the earlier study (*1*). This similarity in viral susceptibility of transformed cells and normal cells contrasts markedly with the work of Ginder and Friedewald (*11*) who showed that Semliki Forest virus multiplied in cells infected with fibroma or myxoma viruses, but was unable to multiply in normal rabbit tissues.

Each of the 6 viruses which multiplied in both the Rous cells and the adult chick cells also exerted a cytopathogenic effect upon the infected cells. This cytopathogenic effect became evident in both types of cells at approximately the same time, depending upon the extraneous virus used. The destruction of malignant cells eventually resulted in loss of tumor-inciting activity by the culture fluids or tumor cells, except for vaccinia virus. Although this agent showed cytopathogenicity, the cellular destruction was incomplete after 16 days and thus the cells and fluids from these cultures maintained their tumor-inciting ability throughout the experiments.

The increasing failure of the infected tumor cells to cause the rapid drop in culture-fluid *p*H characteristic of Rous sarcoma cells was probably due to the cytopathogenic effect of the extraneous viruses. As increasing numbers of malignant cells were destroyed, the remaining viable cells of the cultures were unable to effect the marked *p*H change.

Although the authors feel that basic comparisons may be made between the Rous cells and adult chick cells cultivated *in vitro*, several important considerations should be kept in mind: *a*) It has been assumed that the fibroblastic adult cells, grown out of the muscle tissue surrounding normal chick wing webs, compose at least a portion of those cells which are transformed *in vivo* by infection with the Rous virus. The evidence presented by Sanford *et al.* (*12*) and Loomis and Pratt (*13*) makes this seem likely. In addition, early attempts to transform these adult chick cells *in vitro* after the method of Manaker (*14*) have been successful. Altered cells similar to those described by Manaker have been obtained. *b*) The adult chick cells showed definite multiplication *in vitro*, while

the tumor cells showed only occasional signs of cell multiplication, particularly in the richer culture medium. However, in subsequent experiments in this laboratory, both adult chick cells and tumor cells have been serially passed on glass through more than 12 passages. The differences in cell multiplication are of importance when attempts are made to compare the growth curves and total extraneous virus production of Rous sarcoma and adult chick cells. *c*) The Rous sarcoma cells were cultured in monolayers directly from the tumor tissue. The adult thick cells grew out of chick muscle tissue and were then trypsinized and laid down in monolayers. Thus the adult chick cells had one more *in vitro* passage than the tumor cells. *d*) As outlined under "Materials and Methods," 4 different culture media were used while the experiments were conducted. Recent reports in the literature, as well as work in this laboratory, have indicated that culture medium is extremely important in determining the occurrence and characteristics of viral multiplication. It is felt that herpes simplex and vaccinia viruses are particularly prone to variation in multiplication, depending on culture media employed (*15*). *e*) Although the Rous tissue was dissected with a conscious attempt to avoid any "normal" tissue elements, it is, of course, impossible to claim that there were never any normal cells in the Rous sarcoma cell cultures. However, the authors believe that this fraction was extremely small, since the great predominance of the cells in these cultures were of characteristic Rous cell morphology.

Since this is the first report of any length concerned with the viral susceptibility of adult-chick muscle fibroblastic cells and Rous sarcoma cells, it is interesting to compare the results with those obtained with chick-embryo muscle fibroblasts. The literature contains many reports of multiplication of viruses in cells derived from minced whole embryos, but there are few reports of virus multiplication in specific chick-embryo tissues. Huang (*16*) showed that chick-embryo muscle fragments supported the multiplication of Western equine encephalitis virus in plasma cultures. The multiplication and cytopathogenicity of Newcastle disease virus in chick-embryo muscle fibroblasts was reported by Gey and Bang (*17*) and Pereira and Gompels (*18*). Stulberg and Schapira (*10*) have reported that herpes simplex virus multiples in chick-embryo fibroblasts derived from skeletal muscle, while the PR8 strain of influenza does not. All of these reports also indicate that multiplication was accompanied by cytopathogenicity. A comparison of these results with the adult-chick muscle fibroblast data fails to show any difference between muscle-derived chick-embryo fibroblasts and muscle-derived adult chick fibroblasts with respect to susceptibility to PR8, herpes simplex, Western equine encephalitis, and Newcastle disease viruses.

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The Biological Effects of Some Podophyllin Compounds and Their Dependence on Chemical Structure. II [1]

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Summary

Since deoxypodophyllotoxin and epipodophyllotoxin are of different origin from those compounds used in our previous work, we confirmed their biological inactivity at 200 mg. per kg. The same inactivity was demonstrated for deoxypicropodophyllin. Introduction of a double bond into the B ring of deoxypicropodophyllin and subsequent formation of α-, β-, and γ-apopicropodophyllin restore the toxicity and biological effect on the dividing cell. The effect is fivefold less than that of podophyllotoxin. The toxicity-stathmokinesis index of these compounds is lower than 2, so that the compounds are unsuitable for therapeutic use. Complete aromatization of the B ring of dehydroahydropicropodophyllin is again followed by the loss of the biological activity.—J. Nat. Cancer Inst. 22: 297–301, 1959.

Our previous paper (*1*) described experiments in rats to establish the relationship between the spatial arrangement of the substituents of the lignan skeleton (derived from podophyllin), the lethal dose, and the mitotic effect. An attempt was made to find the substance least toxic, but maintaining the mitotic effect, and to establish the degree of dependence of the biological activity of these compounds on their constitution. From these results, the present report attempts to establish the groups of the lignan skeleton indispensable for the maintenance of effectiveness and their necessary spatial arrangement. The most striking discovery was that both epipodophyllotoxin and deoxypodophyllotoxin are biologically inactive, though their constitution largely corresponds to α- and β-peltatin-A.

Materials and Methods

The same procedures described previously (*1*) were followed. For all experiments male and female Wistar rats, weighing 150 to 200 gm., were used. They were given a single subcutaneous injection of a water-alcohol

[1] Received for publication July 23, 1958.
[2] The authors wish to express their sincerest appreciation to Dr. J. L. Hartwell, Bethesda, Md., for the critical and careful perusal of the work, and to Mrs. I. Bartošová for the translation of this report.

or propylene glycol solution of the compound. The results were identical in both cases. Experiments on controls were carried out simultaneously with the same amount of solution.

The LD50 for each dose was determined in 30 rats, according to the method of Trevan (*2*), and the LD100 in at least 10 rats for each dose. Autopsies were performed and the parenchymatous organs prepared for histologic evaluation (they were fixed in 10% formol, embedded in paraffin, and the sections stained with hematoxylin and eosin).

The stathmokinetic [3] effect was determined by studying regenerating rat livers (*3*). Twenty-seven hours after partial hepatectomy, the substances were injected subcutaneously; 18 hours later, the rats were decapitated and the livers removed and examined histologically for the presence of normal and pathologic mitoses (the stathmokinetic index was calculated from 1,000 liver cells).

The podophyllin derivatives used in our experiments have been identified by the melting point, the optical activity, and the ultraviolet spectrography. Deoxypodophyllotoxin and epipodophyllotoxin, *i.e.*, both our own compounds and those provided by Dr. J. L. Hartwell (*4*), also have been identified by the infrared spectra.

Results and Discussion

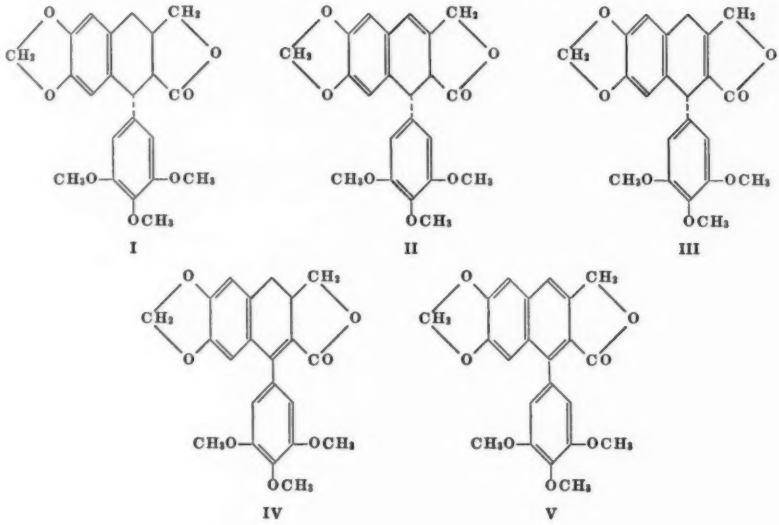
It seemed desirable to verify whether deoxypodophyllotoxin and epipodophyllotoxin, which were of a different origin from those investigated in our previous work, had any biological activity. A good yield of deoxypodophyllotoxin was isolated from resina podophylli in our laboratories (*5*) so that more animals could be subjected to the experiment. Even so, the substance proved to be biologically inactive. Deoxypicropodophyllin (I), a substance which had not been previously investigated, also had no biological activity. The control experiment was carried out with epipodophyllotoxin, which was kindly provided by Dr. J. L. Hartwell; this preparation also did not produce any toxic and mitotic effect when the experiments were repeated. The results confirm our previous findings obtained with these two substances; deoxypicropodophyllin is also biologically inactive.

From picropodophyllin it is possible to prepare derivatives with a partially or completely aromatized B ring, and it is these compounds that we have studied in order to settle definitely the relationship between the constitution and the biological activity of podophyllin derivatives.

Dehydration of podophyllotoxin furnishes α-apopicropodophyllin (II) which is unstable and is converted into β-apopicropodophyllin (III) when treated with alkali. α-Apopicropodophyllin gives rise to γ-apopicropodophyllin (IV), the double bond of which is conjugated with the

[3] As a rule the term "stathmokinesis" is applied to those pathologic forms of mitosis in which, after mitotic arrest of cell division in the metaphase, dispersion of the chromosomes or their fragments is found throughout the cytoplasm, which generally appears enlarged and stains more lightly.

double bonds situated on the C ring. We are indebted to Schrecker and Hartwell (*4*) for the constitution of these compounds.



I II III

IV V

In addition to the loss of the hydroxyl group of the B ring, formation of a double bond and its conversion from α- to γ-apopicropodophyllin is naturally followed by a disturbance in the spatial arrangement of the substituents of this ring. A complete disappearance of the spatial arrangement can be observed in the completely aromatized derivative—dehydroanhydropicropodophyllin (V) (*4*, *6*).

It seemed reasonable to expect that those apopicropodophyllins that have no hydroxyl group but have a second double bond on the B ring would be biologically inactive; these, in addition, undergo a modification from the *trans*(8,7)*trans*(7,6)*cis*(6,5) to the simple *trans*(6,5) configuration or evidence a complete disappearance of this *trans*-isomerism, as with the β- and γ-apopicropodophyllin. But this did not occur. The toxicity and mitotic effect of these derivatives are restored, though the biological activity is fivefold less than that of the starting material, podophyllotoxin. Following administration of these 3 substances there has been slight damage to mitoses. The stathmokinesis index is lower than 2, which suggests that the substances are less suitable for therapeutic use against cancer than podophyllotoxin (table 1).

Dehydroanhydropicropodophyllin, 200 mg. per kg., was also nontoxic in rats.

On the basis of these observations we may complete our previous study, that introduction of a double bond into the B ring and subsequent formation of α-, β-, and γ-apopicropodophyllin restore the toxic activity as well as the mitotic effect on the dividing cell. Complete aromatization of the B ring is again followed by the loss of this biological activity.

TABLE 1.—*Compounds investigated and values obtained*

Investigated compounds	Melting point	$[\alpha]_D$ $CHCl_3$	Configuration of substituents of B ring	Changing substituents of A, B, and C rings				Results				Toxic-stathm. index*
								Toxicity		Mitotic effect		
				R_1	R_2	R_3	Double bonds of the B ring	LD100 (*mg./kg.*)	LD50 (*mg./kg.*)	Dose (*mg./kg.*)	Stathm. index	
Deoxypodophyllotoxin	174°	−123°	*trans*(7,6)*cis*(6,5)	H	H_2	OCH_3	—		>200	200	0	0
Deoxypicropodophyllin	172°	+33°	*cis*(7,6)*trans*(6,5)	H	H_2	OCH_3	—		>200	200	0	0
α-Apopicropodophyllin	244°	−18°	*trans*(6,5)	H	H	OCH_3	7–8	90	60	60	10	1. 5
β-Apopicropodophyllin	220°	+100°	—	H	H_2	OCH_3	6–7	105	70	60	10	1. 3
γ-Apopicropodophyllin	254°	—	—	H	H	OCH_3	5–6	150	100	110	10	1. 7
Dehydroanhydropicropodophyllin	269°	—	—	H	H	OCH_3	5–6, 7–8		>500	200	0	0

*Calculated by dividing the value for LD100 by that for the mitotically effective dose.

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Evaluation of a Volumetric Method for Measuring Growth of HeLa Cells [1,2]

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Summary

A method for measuring the growth of HeLa cells in metric units of volume is described, and its inherent error is analyzed and compared with that of a modified cell-counting method. The volumetric method was less time consuming and tedious, and more samples could be measured per day. The method was found convenient and practical in experiments concerning the nutrition of HeLa cells. This method probably can be applied to measurements of growth in other cell lines.—**J. Nat. Cancer Inst. 22: 303–312, 1959.**

Volumetric measurements of cell populations have long been applied to erythrocytes and bacterial cells by determining the minimum packed-cell volume after centrifugation. Such methods have not been widely used for measurement of mammalian-cell growth in cultures, despite certain apparent advantages. Waymouth (*1*) applied this principle in a rapid method for measuring the growth of strain L cells in very small Carrel flask cultures. The relative volume of cells was determined by centrifugation in microhematocrit tubes.

During the course of cultural work with HeLa cells, the need for a more rapid and convenient method for measuring growth of these cells became evident. This prompted us to develop a volumetric method giving results in metric units, and to evaluate it statistically for its inherent error in comparison to a cell-counting method.

Materials and Methods

Calibration of volumetric glassware.—In order to minimize systematic errors in both packed-cell volume and cell-counting methods, volumetric glassware was recalibrated. The 2 hemacytometers used in counting were certified by the National Bureau of Standards. Volumetric flasks were calibrated by weighing the volume of distilled water contained to the

[1] Received for publication August 11, 1958.

[2] This work was aided by contract NR 135-186 between the Office of Naval Research, Department of the Navy, and Oregon State College.

nearest 0.1 mg. A group of flasks was selected in which the variation from the mean volume was not greater than ±1 percent.

The Van Allen thrombocytocrit tube [3] (*2*) was found to be adaptable to the measurement of the packed-cell volume of HeLa cells. This tube consists of a glass capillary with an overlying bulb of 7 to 8 ml. capacity. The capillary stem has a total volume of 0.03 ml. and is graduated in divisions of 0.0005 ml.

In order to adapt these tubes for the required purpose, a calibration mark was etched on the stem of each tube above the bulb, and the total volume of the bulb and graduated stem below was determined by weighing the tube when it was dry, and again when it was filled with distilled water. The volume of the graduated stem was determined by filling with Hg and weighing the latter. A group of 8 tubes was selected in which the variation of stem volumes was at a minimum; *i.e.*, all stem volumes were within ±1.64 percent of the mean. These tubes were used for all cell-volume measurements.

Cell-culture methods.—HeLa cells were cultivated in 150 ml. pyrex dilution bottles, by the same general methods described by Syverton *et al.* (*3*). However, the nutrient medium employed routinely contained lactalbumin hydrolysate,[4] yeast extract, dextrose, balanced salt solution, and human serum. It was similar to the medium described by Melnick (*4*). The solutions had the following composition:

Solution LAH1 (sterilized by filtration)	Gm. per liter
NaCl	6. 80
KCl	0. 400
$CaCl_2 \cdot 2H_2O$	0. 265
$MgSO_4 \cdot 7H_2O$	0. 200
$NaH_2PO_4 \cdot H_2O$	0. 125
Dextrose	4. 50
Lactalbumin hydrolysate	5. 00
Phenol red	0. 0125
Distilled, deionized water to final volume of 1 liter.	

Solution YE (sterilized by filtration)	
Yeast extract (Difco)	20. 0
Dissolved in Hanks' balanced saline and diluted to 1 liter.	

When required for use, a mixture of 85 percent by volume of solution LAH1, 5 percent of solution YE, and 10 percent of human serum was made aseptically. Penicillin and streptomycin were incorporated at 100 units and 100 μg. per ml., respectively.

For routine culture transfers, cells were removed from the glass surface by exposure to a 0.25 percent solution of trypsin [5] in Hanks' balanced saline, which had been adjusted to *p*H 7.5, sterilized by filtration, and held in the frozen state at about −20° C.

[3] A. H. Thomas Co., Philadelphia, Penna.
[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Difco trypsin 1:250 from Difco Laboratories, Inc., Detroit, Mich.

Cell suspensions for counting were prepared with buffered saline solution of the following composition:

NaCl	8.50 gm.
M/15 KH_2PO_4 solution	38.0 ml.
M/15 Na_2HPO_4 solution	62.0 ml.
Phenol red; 0.2 percent aqueous solution	10.0 ml.
Distilled, deionized water to make 1 liter.	

This solution was sterilized by autoclaving at 122° C. for 15 minutes. The *p*H was 7.0 ± 0.05.

Volumetric measurement of HeLa cells.—Each Van Allen thrombocytocrit tube had a spring sealing clip. Rubber sleeves about ¼-inch wide were cut from rubber tubing of suitable diameter to fit snugly around the bulbs, to cushion them from the walls of the 50 ml. centrifuge cups. A very thin coat of white petrolatum applied to the lower end of the graduated stem prevented leakage between the latter and the hard rubber liner of the sealing clip. The inside surface of the bulb of each tube was coated with Desicote [6] before the study began. This was important to prevent adherence of some cells to the glass surface.

The measurement of the packed-cell volume of a culture of HeLa cells was carried out as follows: The nutrient fluid was first drained away and removed with a pipette. Ten ml. of buffered saline was added, and the cells removed from the glass with a rubber policeman and suspended in the saline. Clumps were broken up by drawing into and expelling from a fine-tipped 10 ml. pipette at least 4 times, and the suspension was transferred to a 25 ml. volumetric flask. The culture bottle was rinsed twice with 5 ml. portions of saline, which were added to the flask; the contents were then diluted to volume with saline. Mixing was accomplished with a 10 ml. syringe and 19-gauge, 2½-inch needle by filling and emptying the syringe 3 or 4 times.

A Van Allen tube was then filled from the syringe by inserting the needle in the upper stem and carefully filling the bulb. The lower stem was filled by tipping the sealing clip slightly to allow replacement of entrapped air by cell suspension. The tube was filled to the mark on the upper stem and prepared for centrifuging by inserting the lower end into the center well of a rubber-hood-type stopper of the proper diameter to fit snugly into a 50 ml. centrifuge cup. This stopper replaced the ordinary rubber cushion and also served to hold the stem of the Van Allen tube in the center of the cup. The rubber sleeve was adjusted around the outside of the bulb, and the tube placed in the centrifuge cup. In the horizontal head of the International size 2 centrifuge, 8 tubes can be accommodated in a single run. The tubes were centrifuged at about 2300 rpm (approximately 1000 × g) [7] for 10 minutes. At this point a 19-gauge needle on a silicone-coated 10 ml. syringe was introduced into the bulb of each tube and about three fourths of the fluid was withdrawn. With the needle directed against the tapered lower part of the bulb, the fluid

[6] Beckman Instruments, Inc., Fullerton, Calif.

[7] Calculated for 16.35 cm., the average radius of rotation at the midpoint of the tube.

was forced back into the bulb, while the tube was rotated. This served to resuspend a fine film of sediment that tended to accumulate at the base of the bulb just above the graduated stem. The tubes were then centrifuged at 3500 rpm (2240 × g)[8] for 45 minutes to give the minimum packed-cell volume, which was read directly in units of 0.0005 ml. from the graduated stem. Increasing the time of centrifuging beyond 45 minutes did not reduce the volume further. The volume of the cell suspension contained in the Van Allen tube was known, and thus the packed-cell volume could be corrected to read in terms of any desired volume of cell suspension, or of packed-cell volume for an entire culture bottle.

Method of counting HeLa cells.—A modified cell-counting method was used, designed to count whole cells instead of nuclei, and to provide a satisfactory cell suspension free from clumps. A suspension of cells from each culture bottle was prepared in buffered saline, as described for the volumetric method, and diluted to 25 ml. in a volumetric flask. Where both methods of measurement were to be applied to the same suspension, 8 or 10 ml. was withdrawn for the volume measurement. Dry hyaluronidase[9] was added to the remaining suspension in the flask to give a concentration of 2 mg. per ml. The flask was incubated at 35° C. for 15 minutes and placed on a reciprocating shaker with a 3-inch stroke, operating at 320 strokes per minute, for 1½ minutes. This gave excellent dispersion of cells, which were mostly single, although occasional groups of 2 or 3 were seen. Suspensions were counted in certified hemacytometers. The number of cells found in the entire ruled area of 9 mm.^{2} was counted. If this number was more than 400, the suspension was accurately diluted to reduce the number to the range of 200 to 400, and a recount was made. For each count, both sides of the hemacytometer representing an area of 18 mm.^{2} were used. If the total count per 9 mm.^{2} area was less than 200 cells, a second count was made, after the chamber was cleaned and refilled. In this way the number of cells counted from each suspension was within the range of 400 to 800. The number of cells per ml. was calculated from the expression:

$$\frac{\text{Number of cells per 18 mm.}^2}{\text{Volume of sample (or 1.8 mm.}^3\text{)}} \times 1000 = \text{Number of cells per ml.}$$

Results

Analysis of Volumetric and Cell-Counting Methods

In order to obtain an estimate of the inherent error in the packed-cell volume method of measuring cell growth, and to compare it with the modified counting method, a series of 34 pools of HeLa cells suspended in buffered saline was prepared in the manner described. Duplicate measurements of each pool were made by both volumetric and counting methods. The concentrations of cells in these pools varied over an approximately 18-fold range. The analysis of error for the volumetric measurements and for the cell counts is shown in table 1.

From these data it may be seen that the mean of the series of packed-

[8] *See* footnote 7.

[9] Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 1.—*Analysis of inherent error in volumetric and counting measurements of HeLa cell growth, and of variation in volume of replicate cell cultures*

Source of data	Mean values	Standard deviation	Least significant difference* between 2 measurements	Coefficient of variation (*percent*)
Duplicate volumetric measurements on 34 suspensions of HeLa cells; expressed as ml. per 10 ml. of cell suspension	13.64×10^{-3}	$\pm 0.86 \times 10^{-3}$	2.44×10^{-3}	6.33
Duplicate counts of 34 suspensions of HeLa cells; expressed as cells per ml.	358.9×10^{3}	$\pm 14.5 \times 10^{3}$	41.0×10^{3}	4.05
Single volumetric measurements on 24 replicate HeLa cultures; packed-cell volume in ml.† per culture	35.67×10^{-3}	$\pm 4.61 \times 10^{-3}$	13.04×10^{-3}	12.92

*Difference between single measurements on 2 experimental treatments that must be exceeded before chance factors can be ruled out ($P = 0.05$).
†Values are also in terms of ml. of packed cells per 25 ml. of cell suspension, as all cultures were accurately made up to this volume.

cell volume measurements was 13.64×10^{-3} ml. per 10 ml. of cell suspension, and the standard deviation was $\pm 0.86 \times 10^{-3}$ ml. Thus, any single measurement was determined with 95 percent confidence within a range of $\pm 1.82 \times 10^{-3}$ ml. The standard error of the difference between 2 measurements was 1.22×10^{-3} ml. Hence, single packed-cell volume determinations on each of 2 experimental treatments must differ by more than twice this value, or more than 2.44×10^{-3} ml. per 10 ml. of cell suspension in order for this difference to be considered significant ($P = 0.05$). The coefficient of variation, which is the standard deviation expressed as percent of the mean, was found to be 6.33 percent. This provides an index of variation in the method for comparison with other methods.

The mean of the series of cell counts was 358,900 cells per ml., and the standard deviation was $\pm 14{,}500$. Thus a single count was determined with 95 percent confidence within a range of $\pm 30{,}600$ cells per ml. The standard error, 20,500, of the difference between 2 counts means that the difference between single counts on each of 2 experimental treatments must exceed 41,000 cells per ml. in order to be considered significant. The coefficient of variation was 4.05 percent.

It seems apparent from these data that the inherent error or variability in the two methods is similar. This is seen most easily by a comparison of the coefficients of variation. The small difference between the two values appears to be insignificant. From this standpoint, then, the volumetric method seems to be equivalent to but not better than the modified cell-counting method described.

The two methods were also compared with respect to economy of time. If only a single measurement were to be made, the cell count was more rapid, requiring about 34 minutes compared to about 69 minutes for the packed-cell volume. However, if 8 or more measurements were to be made concurrently, the average working time for a single measurement was about 9 minutes for the volumetric method and 16 minutes for the cell count. The elapsed time for 8 measurements was about 2 hours in both cases. However, with the volumetric method, the worker was free to do other work during almost half of this period. Another advantage of this method was the fact that less skill and experience were required on the part of the worker. The work was also less tedious where a number of measurements were required on the same day.

The relationship between the volumetric and counting methods was studied by regression analysis (*5*) and found to be linear. The equation of the regression line, found by the method of least squares, was $Y = 2.149\,X + 53.43$ where Y is the cell count in thousands per ml. and X is the packed-cell volume in units of 0.0001 ml. per 10 ml. of cell suspension. From this relationship, the cell count may be predicted for any known value of the packed-cell volume. However, the standard error of each prediction is large; *e.g.*, at the mean it is $\pm 70.4 \times 10^3$ cells per ml. Thus, the predicted value would be unlikely to differ from the true value by more than $\pm 140.8 \times 10^3$ cells per ml. ($P = 0.05$).

Total Variation in Growth of Replicate HeLa Cell Cultures

In addition to the sampling errors inherent in methods of measuring growth, replicate cell cultures treated identically, as far as possible, will show individual differences in growth. In order to obtain an estimate of the total variation between measurements, contributed both by the cultures and the method of measurement, a series of 24 culture bottles was inoculated with carefully measured aliquots of a fresh cell suspension equivalent to about 0.0026 ml. of packed cells per bottle. The bottles were incubated for 4 days, the nutrient fluid was replaced, and incubation continued until the 8th day. Growth in each bottle was then measured by the packed-cell volume method.

Analysis of the variation found is shown in table 1. The mean packed-cell volume per culture was 35.67×10^{-3} ml. The standard error of the difference between 2 cultures was 6.52×10^{-3} ml., and, to be judged significant, a difference greater than twice this value or greater than 13.04×10^{-3} ml. must be found between single cultures on 2 experimental treatments. The coefficient of variation for this series of replicate cultures was 12.92 percent. When compared with the corresponding value for the volumetric measurement alone, the additional variation contributed by the cultures themselves is evident.

Estimation of Mean Volume of Single HeLa Cells

The packed-cell volume measurements and the cell counts on the 34 suspensions of HeLa cells provided the necessary data for estimating the mean volume of single cells. The growth period of cultures from which these suspensions were prepared varied from 4 to 8 days. By dividing each packed-cell volume measurement by its corresponding cell count, we obtained a value for the mean cell volume. The mean of all 34 of these values gave 3.75×10^3 μ^3 as an estimate of the volume of the average HeLa cell, which is approximately 42 times the mean volume of the human erythrocyte in the adult (*6*). This estimate is in remarkably close agreement with that obtained by Puck *et al.* (*7*) with an entirely different method. By microscopic measurement of the diameters of spherical cells resulting from mild trypsinization, they found a mean cell volume of 3.7×10^3 μ^3 for cells grown in a medium containing human serum. By the packed-cell volume method described here, the range of individual values obtained extended from 2.3×10^3 to 6.0×10^3 μ^3. No clear-cut relationship could be observed between the age of the culture and the mean volume of the cells.

Use of Volumetric Method for Measuring Growth in Comparison of Nutrient Media for HeLa Cells

It was of interest to compare the amount of HeLa cell growth obtained in several nutrient media by the volumetric method of measurement. Obviously replicate cultures on each medium were desirable to reduce the error involved in measurements and to make possible the detection of

smaller differences than would be possible with single cultures. For the latter, as previously shown, a difference greater than 13.04×10^{-3} ml. between cultures would be necessary to be judged significant. With cultures whose total cell volumes are of the order of 6×10^{-3} to 40×10^{-3} ml., the above difference represents a large percentage of increase or decrease.

In theory, provided the underlying assumptions are satisfied, the difference necessary for significance can be decreased to any desired point by increasing the number of replicates sufficiently (*8*).

However, practical considerations limit the number of replicates that can be used. With these points in mind, 6 replicates were employed for each medium in the following experiment.

The results of the experiment, with 4 different nutrient media, are presented in table 2. The inoculum of cells for all 24 culture flasks was prepared from a single pool of HeLa cells cultivated in the lactalbumin

TABLE 2.—*Growth of HeLa cells in 4 nutrient media measured by the volumetric method*

Medium*	Culture number	Packed-cell volume per flask in ml.†	Average increase in cell volume within 8 days
#1 10% HuS, 2% CEE, 88% LAH2 (autoclaved)	1	0.0102	
	2	0.0098	
	3	0.0102	
	4	0.0125	
	5	0.0098	
	6	0.0065	
	mean	*0.0098*	4.3-fold
#2 10% HuS, 5% YE solution, 85% LAH2 (autoclaved)	1	0.0092	
	2	0.0125	
	3	0.0105	
	4	0.0120	
	5	0.0120	
	6	0.0150	
	mean	*0.0119*	5.3-fold
#3 10% HuS, 5% YE solution, 85% LAH1 (filter-sterilized)	1	0.0350	
	2	0.0365	
	3	0.0475	
	4	0.0365	
	5	0.0388	
	6	0.0360	
	mean	*0.0384*	16.7-fold
#4 40% HuS, 2% CEE, 58% BSS	1	0.0065	
	2	0.0073	
	3	0.0070	
	4	0.0068	
	5	0.0065	
	6	0.0063	
	mean	*0.0067*	2.9-fold

*All media contained 100 units of penicillin and 100 μg. streptomycin per ml. HuS = human serum; CEE = chick-embryo extract; LAH2 = 5 gm. lactalbumin hydrolysate and 4.5 gm. glucose dissolved in Hanks' balanced saline and diluted to 1 liter. LAH1 differs from LAH2 only in the proportions of the various salts, and in being filter-sterilized rather than autoclaved. Its composition is given in the text. YE = 2 percent yeast extract in Hanks' balanced saline; BSS = Hanks' balanced saline.

†Original inoculum for each flask was approximately 0.0023 ml. in terms of packed-cell volume.

hydrolysate medium and removed from the glass with trypsin solution. After centrifuging and removing the trypsin, we resuspended the cells in a small volume of buffered saline to give a concentrated suspension. The cell content was measured volumetrically, and equal aliquots were added to each of 4 flasks containing 50 ml. of the 4 nutrient media to be compared. Six culture bottles were inoculated from each flask, and incubated at 35° C. On the 4th day the fluid in each bottle was replaced with fresh medium, and on the 9th day the packed-cell volume from each bottle was measured.

It is obvious that medium #3 gave much better growth than any of the others. Medium #2 was sometimes used for routine cell culture because of the greater convenience of autoclave sterilization. Both #2 and #3 contained 5.0 gm. of lactalbumin hydrolysate and 4.5 gm. of dextrose per liter. The proportions of salts differed slightly. In medium #2 these were the same as in Hanks' balanced saline; the salt composition of the LAH1 solution in #3 has been previously defined. The most important difference between the two media appears to be that in #2 the lactalbumin hydrolysate and dextrose solution was autoclaved, while in #3 this solution was filter-sterilized. Apparently the heat destroyed much of the nutrient value of the solution. It seems likely that the heating of amino acids in the presence of glucose may be responsible for this change.

Medium #4, containing 40 percent human serum and 2 percent chick-embryo extract, was commonly employed in earlier work with HeLa cells. However, it gave an even lower yield of cells than #2, containing the autoclaved LAH2 solution and yeast extract.

Discussion

The volumetric method described seems to be a practical and convenient method for measuring growth of HeLa cells. Although it has no greater inherent precision than the counting method, this precision may be increased considerably by using 6 replicates in each experimental group. It is much more practical to use such replication here than with counts, because the volumetric method is less time consuming and far less tedious. It is easily possible for a single worker to measure the growth of 24 cultures in a single day.

We have not attempted to apply the method to measurements of growth of other cell lines, but it seems likely that it would be satisfactory with any cultures, as long as any sedimentable material present could be readily separated from the cells. With cultures grown on glass surfaces this can be done by draining off the nutrient fluid and carefully washing the cell film.

Although we have used the method entirely with relatively small cultures in 150 ml. dilution bottles, it should be equally applicable to much larger cultures. In that event, it would be desirable to redetermine the over-all variation in replicate cultures of larger size.

Insofar as counting methods are concerned, the modification described with hyaluronidase and mechanical shaking to disperse the cells has

given much better cell suspensions, uniformly dispersed and relatively free from clumps, than we were able to obtain without it. In our hands it has been faster and more convenient than the nuclei-counting procedure.

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Mammalian Chromosomes *In Vitro*. X. Heteroploid Transformation in Neoplastic cells [1,2]

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Summary

Three tissue-culture strains were successfully established from Novikoff hepatoma cells originally carried intraperitoneally in Sprague-Dawley rats. The populations *in vivo* consisted roughly of 60 percent s (s = 2n-3 = 39) and 40 percent 2s (2s = 4n-6 = 78) elements. The populations *in vitro* at first showed a decrease in the number of s cells and an increase in 2s cells. Later, the proportion of the 2s group also decreased, and many cells with chromosome numbers in the 60's or low 70's appeared (the stage of spread of chromosome spectrum). After the stage of "spread," the chromosome numbers narrowed and the populations may be represented by a modal chromosome number or by a small group of connecting numbers. Strain N-1 was characterized by possessing 67 or 68 chromosomes; strain N-3, 59 to 61 chromosomes; and strain N-4, 74 or 75 chromosomes. If we consider the s cells "diploid" and the 2s cells "tetraploid," the route of "heteroploid transformation" *in vitro* taken by Novikoff hepatoma cells may serve as a model for the transformation of normal tissues. Prolonged residence *in vitro* seemed to cause the cells to lose their transplantability and perhaps also malignancy. The cell strains finally failed to produce tumors when reintroduced into hosts. These hosts developed a certain degree of immunity against subsequent challenges of tumor cells *in vivo*. Cultivating tumor cells *in vitro* thus appeared to attenuate the virulence of the cells without causing them to lose their antigenicity.—**J. Nat. Cancer Inst. 22: 313–339, 1959.**

It has been found that most normal mammalian tissues transform from diploidy to heteroploidy after cultivation *in vitro*. In a number of cases (*1–3*) structural changes, in addition to numerical increase of chromosomes, also were noted. The phenomenon of transformation has been interpreted as the result of selective adaptation of competing genotypes (*2, 4*). It seems as if some heteroploids, because of their possible metabolic superiority over the diploids in the new environment *in vitro*, may

[1] Received for publication August 12, 1958.

[2] Supported in part by grant DRG-269C from the Damon Runyon Memorial Fund for Cancer Research, Inc., and by grant P-133 from the American Cancer Society, Inc.

[3] We wish to acknowledge the technical assistance of Miss Sally Gardner and Mrs. Lillian Milofsky.

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be selected to replace the diploid genotype that is superior for the condition *in vivo*.

Theoretically, heteroploids may not always be produced in a given population. Those formed may not necessarily possess metabolic advantages over the diploids or tetraploids. Some chromosomal combinations may give the cells disadvantages or may even be lethal. It is also highly possible that heteroploids, after gaining metabolic superiority, may lose the advantages by further recombination of their genetic material. Indeed, Syverton (*5*) reported on a strain of human tissue in which only diploids were found in many transfers. Ford and Yerganian (*6*), in their studies on Chinese hamster cells, found that the diploids coexisted with the heteroploids for long periods. In a cell strain established by Westwood (*7*), it was found that the population containing a high proportion of heteroploids reverted to one containing a high proportion of diploids.

Neoplasms have long been known to possess altered chromosomal patterns *in vivo*. In a recent report on the chromosomal analysis of 60 primary reticulum-cell neoplasms (*8*), for instance, only a few were morphologically indistinguishable from the normal mouse karyotype. Numerous attempts have been made to cultivate neoplasms in tissue culture and many have been successful. The heteroploid constitution of a number of established neoplastic cell strains has been reported (*9*, *10*), but as far as we are aware, no attempt was made to determine whether there was any chromosomal change when cancer cells were cultivated in long-term tissue cultures.

In order to investigate this possibility we have sought a tumor possessing a number of characteristic marker chromosomes that would enable us to trace the cytological history of a cell strain. The Novikoff rat hepatoma (*11*, *12*) was found to be favorable for this type of investigation. The present article presents the results of our observations on the Novikoff hepatoma cells grown in tissue culture.

Materials and Methods

The stock tumor of the Novikoff hepatoma was initially supplied by Dr. Alex B. Novikoff, of the Albert Einstein Medical College, New York, and was carried intraperitoneally in male Sprague-Dawley rats maintained at the Department of Biochemistry of our Institute.

Fresh tumor tissues were obtained 5 days after transplantation, and a cell suspension was prepared by pressing the excised tumor through a 5 ml. syringe equipped with a 30-mesh wire screen. The cell suspension was then introduced into Blake bottles or T flasks for primary cultures without the use of a plasma clot. Eagle's basic medium with 10 percent horse serum was at first used, but it was found later that medium containing 20 percent horse serum was better for the initial cultures. Medium was usually changed 2 to 3 times weekly or as necessary, as indicated by *p*H changes. Subculturing was performed by discarding the used medium, adding fresh medium, and scraping the cells from the glass with the aid

of a rubber policeman. The cells originally suspended in the medium were thus discarded in every transfer. Trypsin or Versene was never used to dislodge the cells. Different containers of the same line were not mixed in seeding, to avoid microbial contamination, except when the containers were too thin for subculturing. Thus, a sample bottle taken from a strain for chromosomal studies may or may not represent the karyological picture of other bottles at that particular time. The time interval for subculturing varied, depending on the rate of cellular growth and on the demand for experimental material. Therefore, both the S (subculture) number and the number of days *in vitro* will be given in the results.

Each group of initial cultures derived from 1 tumor sample was given a number, which later became the symbol for the cell strain. Seven such strains were initiated, but 4 of them were lost due to contamination or to loss of growth vigor of the particular line.

Preparations for cytological observations and idiograms were made as described previously (*1*).

Results

Chromosomes of the Tumor In Vivo

In the ascites populations *in vivo* there were 2 modes of chromosome-number distribution, one at 39 (s) and the other at 78 (2s). From the 2 samples observed (*12*), high polyploids occupied about 1 to 2 percent, 2s cells nearly 40 percent, and s cells the remaining fraction (text-fig. 1)

The s cells have a special karyotype in which are found a number of chromosomes new to the normal rat somatic complement. A brief description of the 6 groups of the most easily recognizable chromosomes in the Novikoff hepatoma cells *in vivo* follows:

Chromosome A.—An extremely long metacentric chromosome, apparently the result of a translocation or fusion of 2 chromosomes. It is almost twice as long as the longest telocentric chromosome (chromosome C).

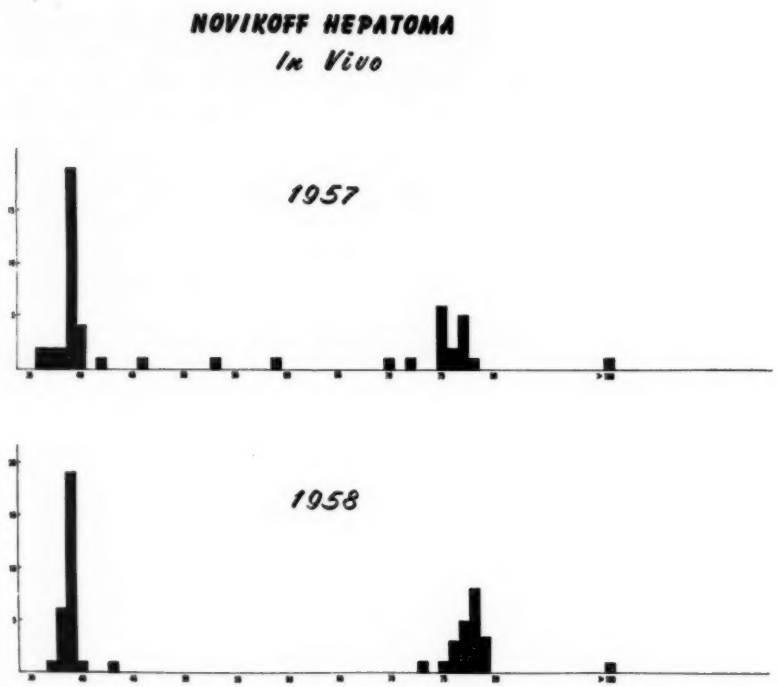
Chromosomes BB.—Two metacentrics differing slightly in length. In colchicine-treated cells it is often difficult to distinguish the 2 elements unless exact measurements are made of the chromosomes lying perfectly flat on the plane of observation. It should be borne in mind that the 2 B chromosomes are not homologous.

Chromosome C.—A pair of long telocentric chromosomes, possibly representing the longest pair of telocentrics in the normal rat karyotype.

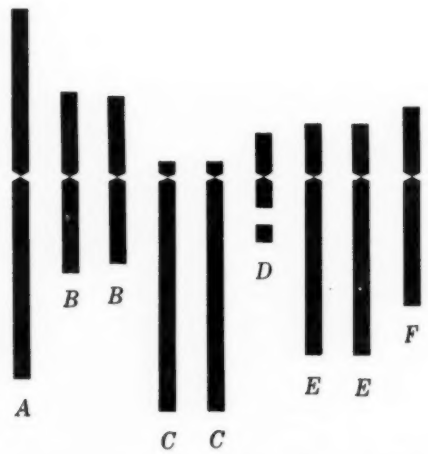
Chromosome D.—A single, small chromosome with a secondary constriction that is slightly longer than the centromeric constriction. This chromosome, in preparations pretreated with colchicine, usually appeared like 3 beads strung together.

Chromosome E.—Two long subtelocentrics, possibly a pair, and possibly representing the pair of the longest subtelocentrics in the normal rat karyotype. The second arm of these chromosomes is relatively short.

Chromosome F.—A single, long submedian chromosome slightly shorter than the E chromosomes.



TEXT-FIGURE 1.—Distribution of chromosome numbers in 2 samples of Novikoff hepatoma *in vivo* (original data from Hsu, *12*).



TEXT-FIGURE 2.—A diagrammatic idiogram for the 6 groups of most easily recognizable chromosomes in cells of the Novikoff hepatoma *in vivo*.

A diagrammatic idiogram for these chromosomes is presented in text-figure 2. Other chromosomes are of various length and shape (*cf.* fig. 12). However, all the short elements are metacentric.

Strain N-1

Cultural and cellular characteristics.—The N-1 line was initiated December 10, 1956. It grew rather slowly and the first subculture was made in about a month. After 6 months the growth rate of this strain increased to such a point that subculture could be made every 7 to 10 days.

The N-1 cells generally remain single or form small colonies that attach to the bottom of the container, and thus present a "powdery" appearance on gross inspection. Microscopically, the cells rarely flatten as do most of the epithelial cells in tissue culture. Usually they appear more or less spherical with a few thin and short-foot processes. Occasionally, at the periphery of the colonies, cells may possess ruffling membranes. A number of the cells contain vacuoles of various sizes resembling "signet rings." Sometimes the signet ring becomes so large that the entire protoplasm is displaced to a corner of the cell. Signet-ring cells usually appear 36 to 48 hours after seeding and will increase in number as cultures grow older. This characteristic morphological feature has persisted since the establishment of this line. A low-magnification photomicrograph is presented in figure 1, which shows the cellular and cultural characteristics of strain N-1.

During the first half-year of the strain's history, several successful attempts were made to isolate clones from this line in 60-mm. petri dishes without feeder layer. The initial colonies were carefully removed from the dishes, resuspended, and inoculated into separate containers. Observations of cultural characteristics were made for the first few subcultures. Cells from some clones exhibited rather broad membranes and formed loose colonies (fig. 2), while those of others formed compact masses with little membrane activity (fig. 3). The clone lines were reluctantly discarded after a few passages so that stock cultures could be maintained within workable limits.

Chromosomal characteristics.—The first attempt to study chromosomes of strain N-1 was made on S-6, day 163, at which time the strain was already well established. A count of 50 cells (table 1) revealed approximately 10 percent of the cells in the s zone and nearly 80 percent of cells in the 2s zone. One week later, the next subculture (day 169) exhibited a cell population in which chromosomes deviated more from the pattern *in vivo* than S-6, although there were more s cells in this sample. A number of elements, with chromosome numbers in the high 60's and the low 70's, were present. The difference between subculture 6 and 7 could not reasonably be due to an additional week of incubation *in vitro*. More likely it was due to sample variation, viz., the bottle for subculture 7 happened to contain more heteroploid cells than that for S-6. As previously mentioned, during routine transfers not all containers of the same strain were mixed when seeding, so that one container might have more changed cells than another.

On day 221, a sample of S-13 was again examined (table 1). In this population there was a significant increase of the heteroploid cells with new chromosome numbers. However, both the s and the 2s representatives were still present. From S-7 to S-13, there was an increasing spread of the chromosome spectrum. Since the s and the 2s cells were already heteroploid in respect to rat karyotype, cells with deviation of chromosome numbers from the stemline composition are therefore secondary heteroploids.

The most remarkable data for strain N-1 were obtained from S-22, at day 283. In this population all the cells were secondary heteroploids with the s and 2s elements completely lacking. The modal chromosome numbers at that time were 69 to 70. After a few months, a sample of S-37 (day 401) was examined to determine the stability of the strain after it had changed. This population showed a further shift of the modality to 67, and also a number of cells in the 2s zone. Again this might be the result of sampling differences. The container for sample S-22 might contain all secondary heteroploid cells, while other containers consisted of mixed cell types. Another possible explanation for the partial reversion to the 2s zone could be that heteroploids, owing to their abnormal mitotic divisions, produced filial elements with near-2s chromosome number. The last sample taken from S-49 (day 484) showed a distribution pattern similar to the previous one.

Even in the earlier samples, such as S-6 and S-7, a number of the 2s cells already possessed a single chromosome A instead of the expected 2. Other chromosomes also showed variation in numbers. For example, BB chromosomes were seen to vary in number from 2 to 6 per cell (expected 4), and chromosome D, from 1 to 3 (expected 2). In the clones isolated during that period, some contained a single chromosome A and others, 2 such chromosomes. Figure 5 shows a cell (S-6) that still retained 2 A chromosomes. In the later generations analyzed (*cf.* fig. 6), few cells contained more than 1 chromosome A.

Complete idiograms are presented for 2 cells taken from S-49 (figs. 11 and 12). A single chromosome A was noted in both cells. There were 4 B chromosomes (the 2s number) and 1 D chromosome (the s number) for each cell. However, the cell in figure 11 contained 5 C chromosomes, while that in figure 12 contained 4 (1 longer than the other 3). It was relatively difficult to discern chromosomes E and F. One reason for this difficulty was that a new type of long subtelocentric chromosome had appeared, which confused the identity of the E and F chromosomes. The long arm of this new chromosome was as long as that of chromosome A, and its short arm was similar in length to that of chromosome F. It is highly possible that this element represented the remainder of the missing chromosome A, which might have had a breakage in 1 arm. In figure 11, 2 minute chromosomes were also recorded. Most of the cells in this population seemed to retain 3 or 4 B chromosomes, but the C chromosomes varied from 2 to 4. In all cells, including those reverted to 2s zone, a single A chromosome and the new, long subtelocentric chromosome were present.

It should be emphasized that variation in the combination of chromosomes was even greater in the samples of S-7 and S-13 than in that of S-37 and S-49, when the population was in the stage of "spread." Perhaps a number of inferior genotypes present at that time were later eliminated from the cell strain after ruthless selection.

In summary, cells of strain N-1 of the Novikoff hepatoma appeared to undergo a gradual process of cytological transformation within a period of approximately 1 year. The population after transformation consisted of cells with chromosome numbers in the high 60's, but many cells were different from each other in their chromosomal combinations.

Strain N-3

Cultural and cellular characteristics.—Strain N-3 was initiated, March 21, 1957, in a Blake bottle. Growth was poor and on several occasions the strain was almost lost. The cells tended to detach from the glass surface. When the culture medium was changed or when subculture was made, the suspended cells were poured off with the used medium. However, even with this constant selection, the cells in new subcultures still became easily detached. The strain grew slightly more vigorously later than during its first 8 months.

The morphological and colonial characteristics of strain N-3 were quite different from those of strain N-1. The cells did not form small, isolated colonies. Instead, they formed rather complex network patterns. Individual cells of N-3 appeared more or less elongated, or even spindle-shaped, as if they were of connective-tissue origin. Rarely were there wide membranes or cells with signet rings. Figure 4 represents a typical field of N-3 cells grown in a Blake bottle.

Chromosomal characteristics.—As early as the third subculture, 119 days after the initial culture, the populations of this strain already contained a very low proportion of the s cells (table 2). Most of the cells were in the sub-2s zone with 74 as the modal chromosome number. Whether the s elements were selected out by the poor growth and the tendency of cells being suspended was not known.

The sample taken from S-9, 181 days *in vitro*, showed a great spread of the chromosome spectrum, ranging from the 60's to the 80's. However, the majority of the cells still retained the chromosome numbers in the low 70's. The most dramatic shift of chromosome number in strain N-3 was noted in S-14, a sample taken on day 307. In this population practically all the s and the 2s components had vanished, and the modal chromosome number was reduced from the low 70's to the low 60's—a drop of 14± chromosomes from the 2s value. The situation was very similar to the shift in strain N-1 witnessed in sample S-22. Unlike strain N-1 in which the population produced some 2s elements after a complete transformation, strain N-3 retained its low chromosome number as shown in the sample of S-26 (table 2), 383 days after its primary culture. In S-26, the modal chromosome number was further reduced to 60 (59–61) and the spectrum was also extremely narrow (58–64).

TABLE 1.—*Distribution of chromosome numbers in Novikoff hepatoma strain N-1—initial culture* in vitro *set up December 10, 1956*

Subc. No.	Days *in vitro*	38	39	40	41	42	43	44	56	57	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	>100	Number of cells
6	163			2			2	1	1	1			1									1	1	1	4	2	6	9	2	8	5		1	2	50
7	169	1	2	2	3	2		1								1		1	1	3	4	4	3		1	2	2	4	1	5	2	1		4	50
13	221			3		2					1					4	4	3	4	4	2		1	4	2	2	2	2	1	1	2			6	50
22	283										1				2	3	6	12	10	5	3	2												6	50
37	401										1		1	4	5	15	4	4	3				2	3	1	3	1	1	1					1	50
49	484										2	2	7	5	5	5	7	4		1			1		4	1	2	1	1					2	50

TABLE 2.—*Distribution of chromosome numbers in Novikoff hepatoma strain N-3—initial culture* in vitro *set up March 21, 1957*

Subc. No.	Days *in vitro*	39	40	43	49	54	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	83	87	>100	Number of cells
3	119	1						1	1				1		1	1	1	3		1	6	4	13	5	7	5	1	1					2	50
9	181		1	1				4	7				7		2		1	2		2	6	5	3	5	3	2	1	1	1		1	1	3	49
14	307					1		12	12	6	6	4	1	3	2	6			5			1					1		1				1	50
26	383				1		3			10	7	3	1																				1	50

In the earlier stages of culture of N-3, variation in chromosomal constitution was already evident. Figure 7 is a photomicrograph taken from the S-3 sample. Two idiograms made during that period are represented in figures 9 and 10. In figure 9 the cell contained 75 chromosomes with 1 chromosome A and another long metacentric chromosome slightly shorter than A but definitely longer than the B chromosomes. There were only 2 B chromosomes instead of the expected 4, 3 distinct C chromosomes, 2-D's, 2-E's, and 2 or 3 F's. In the next cell (fig. 10), which contained 77 chromosomes, it was clear that 2 A chromosomes were present. The cell also contained 5 B's, 2 C's, 2 D's, 4 E's, and 3 F's. These idiograms show that even though the cells might still possess the 2s or near-2s numbers, the combination of the elements had already deviated from the original pattern. The long metacentric chromosome, shorter than chromosome A, was possibly the second A in which breakage had occurred in both arms.

After the transformation process, it was noted that in N-3, as in N-1, only 1 A chromosome could be observed in each cell. In a number of cells only 2 B chromosomes were present. Figures 8, 13, and 14 are examples taken from S-26, 383 days *in vitro*.

The cell shown in figure 13 (60 chromosomes and 2 fragments) contained a single A, 2 B, 2 C, probably 5 E, and no D chromosomes. There was also a short telocentric chromosome, which was new to the karyotype for the Novikoff tumor.

In the idiogram represented in figure 14 (62 chromosomes), again there was a single A and 2 B chromosomes. In this cell a D chromosome was found. There were 3 apparent C's, 4 E's, and a single F chromosome.

In summary, strain N-3 went through a transformation process and became heteroploid with $60\pm$ chromosomes. It also showed a narrow spectrum after transformation.

Strain N-4

The primary culture of strain N-4 was set on June 10, 1957. The cultures grew well from the beginning, and there has been very little trouble in culturing this line. Morphologically the cells *in vitro* appeared more or less like the cells of strain N-3.

The karyological composition of the first subculture of N-4 (day 31) was very similar to that of the populations *in vivo* (table 3). In the sample of S-8 (day 100) a significant reduction of the s elements was noted. The period of great spread of chromosome spectrum was recorded at S-10 (day 123). The population, after this period, narrowed its distributional spectrum as observed in samples of S-15 (day 186), S-21 (day 249), and S-25 (day 302). The modal chromosome numbers after the transformation period centered at low 70's, 3 or 4 chromosomes below the 2s number. In the last 2 samples a few cells with chromosome numbers in the 50's or 60's were observed (table 3).

Two idiograms are presented in figures 15 and 16 taken from cells of S-25. The first cell (fig. 15) was not a typical one. It had only 54 chro-

TABLE 3.—*Distribution of chromosome numbers in Novikoff hepatoma strain N-4—initial culture* in vitro *set up June 10, 1957*

Subc. No.	Days *in vitro*	34	36	37	38	39	40	41	42	43	46	51	52	54	56	58	59	60	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	96	>100	Number of cells
1	31		1	3	3	11	1								1				1							1			1	3	2	3	4	2			2			2	42
8	100	1					1	1	1			1										1	1	2		1	2	2	4	5	6	3	7	4	2	1				2	48
10	123					1		3	1	1	1	1	1			1	1				1	1				1	4	3	8	2	7	3	1	4		1				3	50
15	186																								1	2	15	4	5	6	6	6	3						3	51
21	249						1															1	1	2		3	2	6	13	10	8		2							1	50
25	302										1			1				1		1						1	2	14	3	10	5	6	3							2	50

TABLE 4.—*Distribution of chromosome numbers in Novikoff hepatoma strain N-6—initial culture* in vitro *set up June 21, 1957*

Subc. No.	Days *in vitro*	36	37	38	42	43	55	58	59	60	61	62	64	65	67	68	69	70	71	72	73	74	75	76	77	78	79	81	83	84	89	>100	Number of cells
5	88	1	1											1	1	1		2	1		4	5	7	6	7	5	3	1	1			3	50
7	112	1	1		1	1	1		1	1	1		1		2	1	1	1	1	4	8	5	4	2	1				1	1		9	50
10	175			1				1		1	1	2	1	1	1	2		3	4	1	3	10	5	1	1	2			1			8	50

mosomes, with only a single chromosome each of the A, B, C, and E chromosomes. There was a long subtelocentric element that was much longer than the E element. Again, perhaps this was a modified A chromosome. No D chromosome was noted in this cell. In the next cell (fig. 16), which contained 73 chromosomes, again there was only a single A chromosome. The cell also had 3 B's, 2 C's, 2 D's, probably 4 E's, and 2 F's. Note that one of the D chromosomes was longer than usual. There were 3 very long subtelocentrics that cannot be classified as E chromosomes. Two of these chromosomes had relatively long second arms but the third had a short second arm.

Strain N-4 transformed in a similar manner, but the reduction of its chromosome number from the 2s value was slight, only 3 or 4 chromosomes fewer.

Strain N-6

The initial culture of strain N-6 was set up June 21, 1957. The original tissue was the suspended tumor cells in the ascitic fluid instead of mashed solid tumors. The cultures grew fairly well but the strain was lost due to a bacterial contamination.

Three sets of chromosome counts had been made before the strain was lost. Table 4 summarizes the cytological data. The first sample taken from S-5 (day 88) showed a predominantly 2s population. The second and the third samples, S-7 (day 112) and S-10 (day 175), respectively, were both in the stages of spread of chromosome spectrum.

Strain N-7

Strain N-7 was initiated on June 21, 1957, the same date as strain N-6, but the tissues were taken from a solid tumor of another animal. The cultures grew well but were unfortunately lost by a fungus contamination.

Only 2 samples were examined to determine the chromosomal constitution of this strain. The first was taken from subculture 3, 32 days *in vitro*. The population consisted almost entirely of s cells. Among 50 cells examined (table 5), only 3 were in the 2s zone and none was a high polyploid. Many slides of this set were inspected and the above-mentioned proportion was found to be consistent. In S-7 (day 57), however, a significant increase in the number of 2s cells was noted. They accounted for approximately 25 percent of the population. Karyotypic analysis of the population in S-7 showed little change from the constitution *in vivo*. In a number of the 2s cells studied, typical combinations of the 6 obvious

TABLE 5.—*Distribution of chromosome numbers in Novikoff hepatoma strain N-7—initial culture* in vitro *set up June 21, 1957*

Subc. No.	Days *in vitro*	36	37	38	39	40	41	42	43	45	49	75	76	77	78	79	80	81	Number of cells
3	32	1	3	10	26	3		2	1						1	1	1		50
7	57	1	2	5	23	3	1			1		2	2	2	5	1		1	50

groups of chromosomes (2 A's, 4 B's, 4 C's, 2 D's, 4 E's, and 2 F's) were recorded in almost all cases.

Transplantability of Cells In Vitro *in Original Hosts*

Whenever surplus cultures were available, cells from the tissue-culture strains were inoculated either subcutaneously or intraperitoneally into young Sprague-Dawley rats to check their tumor-producing capacity. Other investigators (*13*, *14*) have found that short residence *in vitro* does not alter the ability of transplantable neoplastic cells to produce tumors. Occasionally it has been reported that in long-term cultures tumor cells may lose their malignancy (*15*, *16*). The transplantability of our cell lines into Sprague-Dawley hosts was mentioned in an abstract on the cytological changes of the Novikoff hepatoma cells *in vitro* (*17*). An account of our experiments conducted during the early cultural periods of the Novikoff hepatoma lines is listed as follows:

Strain N-1.—In the 7th month of culture a subcutaneous implant to the rat produced a tumor which, when reimplanted intraperitoneally, produced tumors and caused death of the hosts similar in behavior to the Novikoff tumor implants *in vivo*. In the 8th and the 9th months subcutaneous and intraperitoneal implants grew. In the 10th month a subcutaneous implant "took" but an intraperitoneal implant did not cause death of the host. Two months after the intraperitoneal implantation, the animal was killed and no tumor was found in the body. Subsequent attempts with N-1 cells all failed to produce tumorous growth in the rats.

Strain N-3.—After 5 months of culture, an intraperitoneal implant produced a tumor. In the 11th month, 2 rats received the N-3 cells subcutaneously but no tumor growth was registered.

Strain N-4.—In the 2nd, 4th, and 6th months of culture intraperitoneal implants all produced tumors in the host. But in the 9th month, and in subsequent months, no tumors were recovered upon implantation.

Strain N-6.—In the 2nd and the 4th months of culture intraperitoneal implants produced tumors. The strain was lost at this time.

The hosts, which failed to produce tumors after implantation with the cell strains, became somewhat immune to the subsequent challenges of the malignant tumor cells *in vivo*. In a number of instances the malignant implants did not grow at all; in others they grew to a certain size and then regressed. Also, it seemed that the cells from strain N-1 elicited better immune responses than those from N-3. In only 3 of 15 cases tested did the tumors grow to kill the hosts which had received inoculations of the N-1 cells.

Discussion

In a recent paper (*1*) we reported several cases of contamination of one cell strain with another. Since that time we have been informed of several additional incidents, including a report on a number of such cases by Rothfels *et al.* (*18*). Fortunately, in the strains of the Novikoff hepatoma, the characteristics of the chromosomes, such as the extremely long

A chromosome, the shorter metacentric B chromosome, and the long secondary constriction of the short D chromosome, assisted us in establishing their identity beyond doubt. Not only have the strains remained uncontaminated with established lines from other animals but also they have not been contaminated with normal rat cells that might have been in the initial cultures. The process of chromosomal transformation reported here has been morphologically verified and is not the result of laboratory accidents. This work also suggests that heteroploid transformation did actually take place in cultures of normal mammalian tissues and that such transformation cannot always be regarded as the result of contamination.

Ironically, the cytological follow-up on the Novikoff hepatoma was initiated to test the supposition that its chromosome number would not change drastically as normal tissues usually do, since the Novikoff hepatoma cells were already heteroploid *in vivo*, whether they were s (2n−3) or 2s (4n−6) elements. Furthermore, as has been postulated (*4*), the heteroploid transformation of normal tissues could be a result of repeatedly selecting genotypes that are best adapted to a semianaerobic environment. Selection in this direction may not be effective on Novikoff hepatoma cells since they already possess high activity of anaerobic glycolysis in the *in vivo* state (unpublished data). Contrary to our supposition, all of our Novikoff cell strains changed their chromosomal constitution in a manner similar to some of normal tissues.

If, for the sake of convenience, we call the stemline cells (39 chromosomes) of the Novikoff hepatoma "diploids," the 2s cells (78 chromosomes) "tetraploids," and the cells with chromosome numbers in between these 2 typical numbers "heteroploids," then our data on the 3 established cell lines show that all of them gradually changed to a "heteroploid" condition. However, each of the 3 strains (N-1, N-3, and N-4) followed its own route of transformation. Strain N-1 finally reached a modal chromosome number 10 or 11 below tetraploidy; strain N-3, 18 below; and strain N-4, 3 or 4 below. Had the containers of the same strain been kept strictly separated, it is highly possible that each initial bottle of the same sample would have shown its own course of evolution as if they were different strains. This has actually been demonstrated in Chinese hamster tissue cultures by Ford and Yerganian (*6*).

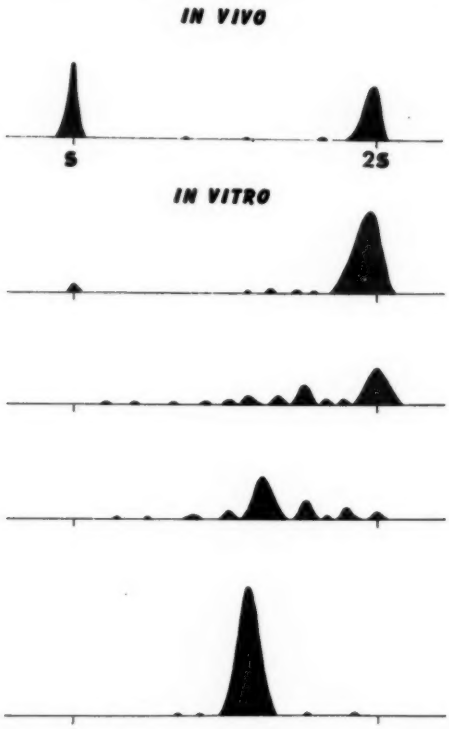
The 2 strains (N-6 and N-7) that were lost before conclusions could be made actually fill in the gap for the earlier stages of transformation. Strain N-7 consisted largely of diploid cells in the beginning. The second sample, taken only 25 days after the first one, already showed an unmistakable increase of the 2s elements. Strain N-6 was terminated at the period of wide spread in chromosome spectrum, which appears to be the essential step toward transformation. This was the stage at which numerous genotypes were present, but only a few of them survived and emerged as dominating elements of the populations.

A survey of tables 1 to 4 for the period at which a wide spread of chromosome number appeared reveals that it was attained at periods

varying from the 3rd to the 7th month after primary culture. The earliest record was found in strain N-4 at day 100, and the latest, in strain N-1 at day 221. If we could have saved strain N-7, it would not be surprising to see the 2s cell gradually increasing in proportion, and later the population reaching a stage of great spread of chromosome number.

According to our available information, the process of transformation may be generalized as follows: The population first makes a shift from diploidy to tetraploidy and then widens its numerical variation to the stage of "great spread," possibly by gaining and losing chromosomes through mitotic anomalies of the tetraploids. Finally, a narrow range of heteroploids may be selected to represent the strain. Text-figure 3 is a diagrammatic representation of this scheme.

Whether or not the process of transformation just described and discussed is characteristic only for the Novikoff hepatoma remains to be determined. Investigations of a similar nature with other animal tumors are under way to test this point. However, it seems possible that the transformation process of normal tissues follows this pattern. Perhaps



TEXT-FIGURE 3.—Diagrammatic representation of population changes in the Novikoff hepatoma cells grown in culture. *Top figure:* proportion of s and 2s cells in the ascites. *Other 4 figures* represent sequential changes that generally take place in the populations *in vitro*.

the difference between Novikoff hepatoma and normal tissue is that most Novikoff cells are more or less adaptable to the environment *in vitro*, thus enabling a gradual transformation procedure; whereas, in the latter case, some heteroploid cells gain great selective and adaptational advantages over diploids and tetraploids, thus replacing the genotypes *in vivo* in a sudden fashion (*19*, *20*).

It is obvious from an inspection of the idiogrammatic examples that extensive chromosomal breakages and reunions have occurred during the history of the Novikoff cell lines. The minutes in figure 11, the acentric fragments in figure 13, the small telocentric in figure 15, and the long basic arm of the D chromosome in figure 16 are strong evidence of such structural alterations. The most remarkable phenomenon, however, is the reduction in the number of A chromosomes. In all 3 established lines, possession of a single A chromosome was observed in the overwhelming majority of cells, even though chromosome numbers varied from strain to strain. It appeared that heteroploid cells with 2 A chromosomes were not stable. Possession of the new subtelocentric chromosome (presumably the other A chromosome with a broken arm) appeared to be more satisfactory for metabolic balance than having 2 intact A chromosomes. In the Chinese hamster cell cultures, chromosome I (the longest chromosome) was found to break more often than other elements (*21*). It would be of interest to to find out why the 2s cells *in vivo* are satisfied when both A chromosomes are present, while in cells *in vitro* this is not found to be the case.

Cytological findings on the Novikoff hepatoma substantiate our concept of flexibility of stem line (*1*, *4*). The s cells with a definite chromosome number and a special karyotype are undoubtedly stemline cells for the populations *in vivo*. From generation to generation, deviating genotypes are formed yet the main genotype persists in, and dominates, the populations. Once the tumor cells are subjected to an adverse environment, such as the condition *in vitro*, the stemline cells may be replaced by other genotypes.

The loss of transplantability in all the Novikoff tumor strains *in vitro* contrasts sharply with the much discussed concept of carcinogenesis *in vitro* in normal tissues. The tumor cells were altered so profoundly after long-term culture that probably they grew quite slowly after reinoculation into the host. This would permit the host time to mobilize defense mechanisms, immunological or otherwise, for destruction of the implants. The situation may be analogous to the cases reported by Sanford *et al.* (*22*), who studied the growth *in vivo* of mouse cell lines which produced low tumor incidence. Though our limited data do not permit much speculation at this time, they do suggest the possibility that cultivation of tumor cells *in vitro* may be of use in attenuating virulent tumor antigens.

Addendum

Dr. H. Katsuta, Department of Pathology, The Institute for Infectious Diseases, Tokyo, Japan (personal communication), has informed us that his 2 lines of ascites hepatoma grown in tissue culture also changed their

chromosomes from near-diploid to heteroploid. However, in one of his publications (T. Takaska and H. Katsuta: Establishment of cell strain of rat ascites hepatoma cells in tissue culture, Japan. J. Exper. Med., 28: 115–127, 1958) it was mentioned that the hepatomas in culture maintained their malignancy after nearly a year's residence *in vitro*.

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PLATE 23

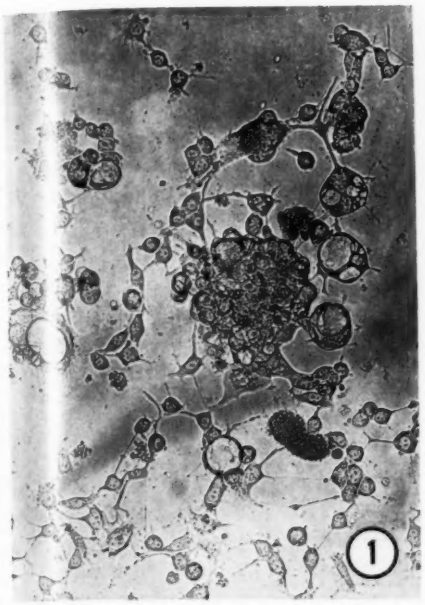
Photomicrographs of Novikoff hepatoma cells grown in tissue-culture vessels. Light microscopy. Figure 1, T-30 flask culture; figures 2 to 4, Blake bottle cultures. Approximately × 240

FIGURE 1.—Strain N-1. *Note* small, roundish cells with thin cytoplasmic processes. *Also note* abundance of cells with large vacuoles (signet rings).

FIGURE 2.—A cell colony of a clone derived from strain N-1. *Note* loose association of cells. Compare with figure 3.

FIGURE 3.—A cell colony of a clone derived from strain N-1. *Note* closely packed cells with little indication of membrane activity. Compare with figure 2.

FIGURE 4.—Strain N-3. *Note* spindle-shaped cells and network pattern of cellular association.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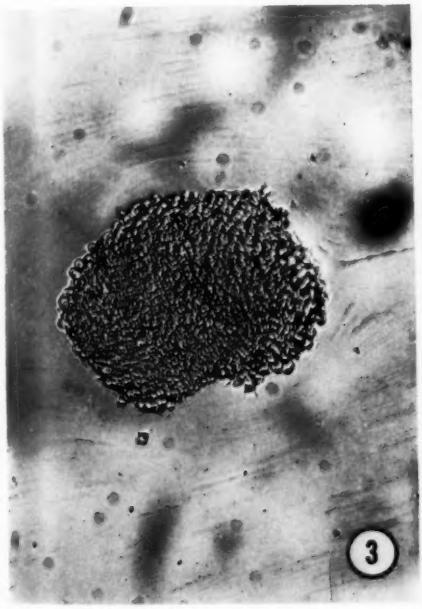
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PLATE 24

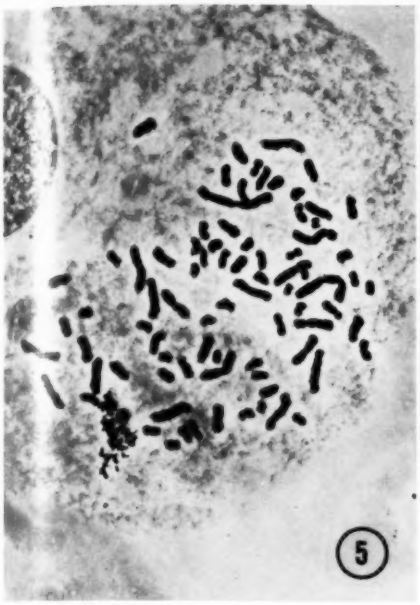
Photomicrographs of Novikoff hepatoma cells showing chromosome constitutions. Phase microscopy. Approximately × 1,100

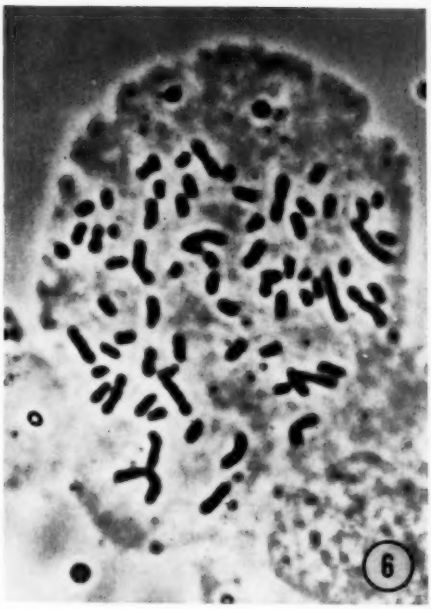
FIGURE 5.—A cell from strain N-1, S-6; 81 chromosomes. *Note* 2 A chromosomes.

FIGURE 6.—A cell from strain N-1, S-49; 71 chromosomes. *Note* a single A chromosome.

FIGURE 7.—A cell from strain N-3, S-3; 73 chromosomes. *Note* presence of a large subtelocentric chromosome.

FIGURE 8.—A cell from strain N-3, S-26; 64 chromosomes. *Note* single A chromosome.





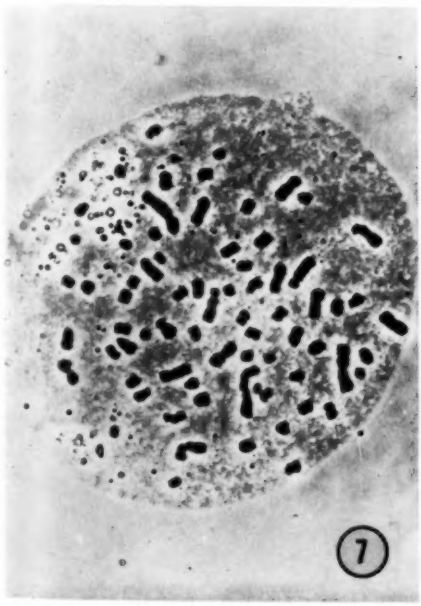


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PLATE 25

FIGURES 9 and 10.—Complete idiograms for 2 cells of strain N-3, S-3.

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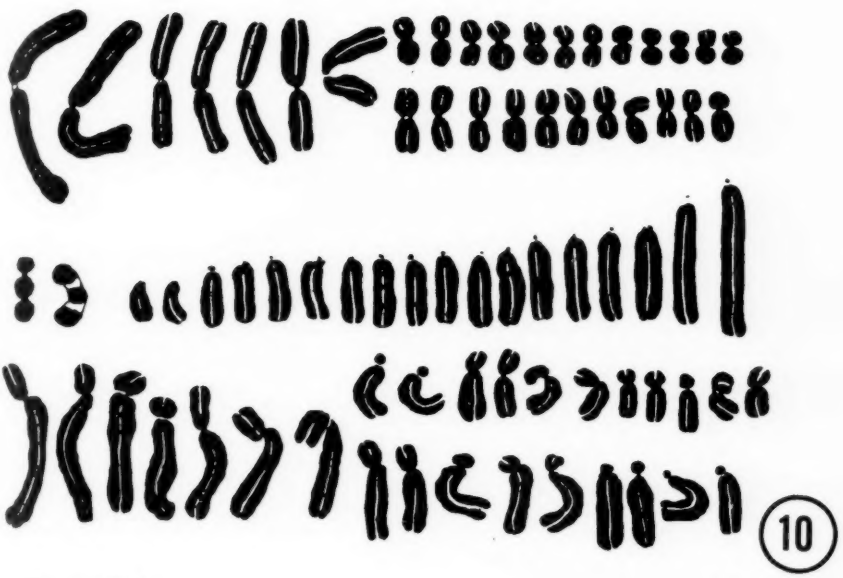
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PLATE 26

Complete idiograms for 3 cells of Novikoff hepatoma strains.

FIGURES 11 and 12.—Strain N-1, S-49.

FIGURE 13.—Strain N-3, S-26.

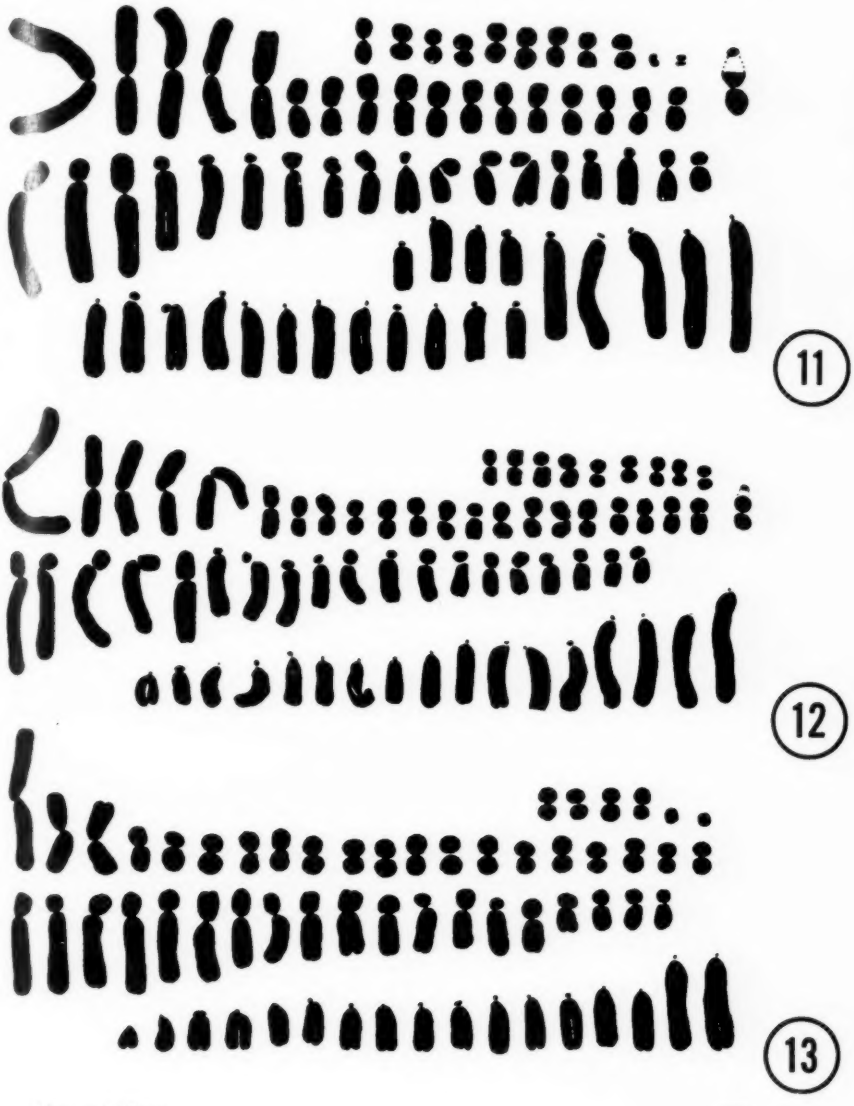
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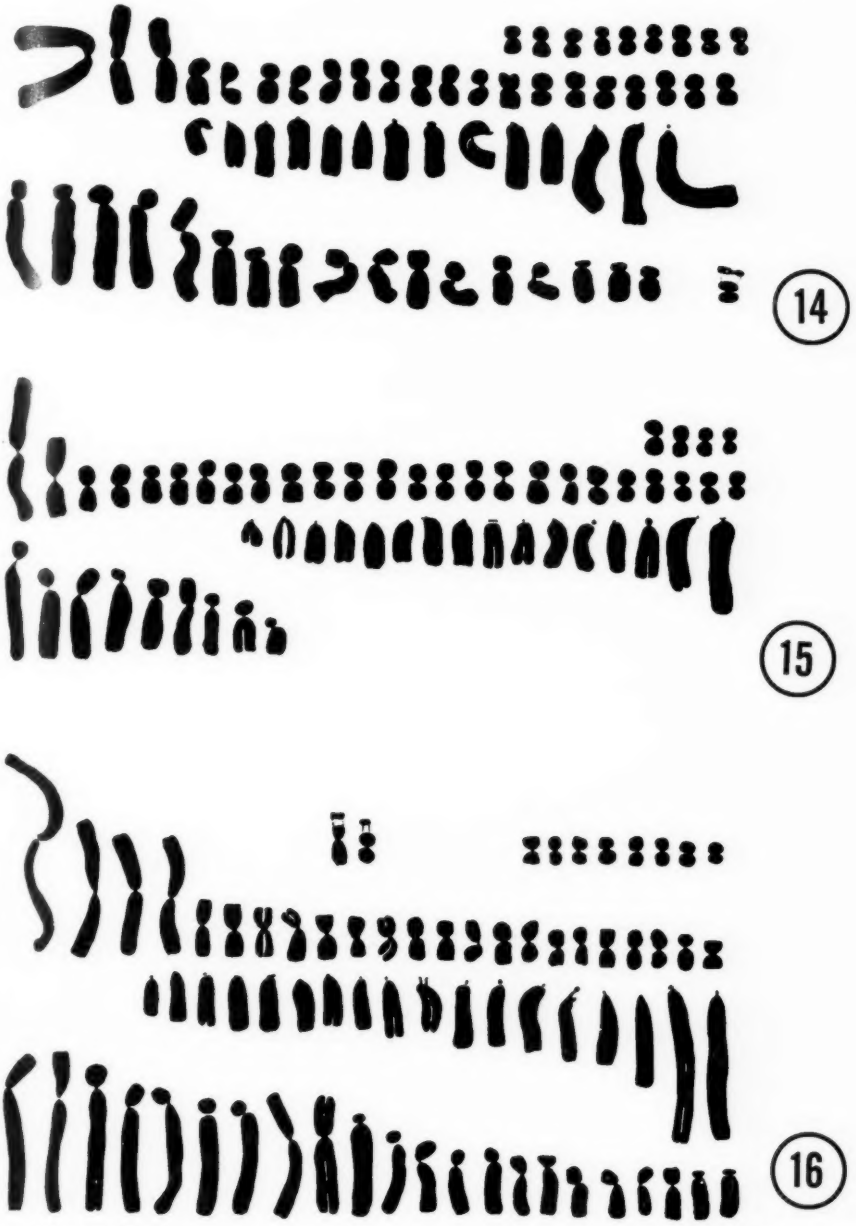
PLATE 27

Complete idiograms for 3 cells of Novikoff hepatoma strains.

FIGURE 14.—Strain N-3, S-26.

FIGURES 15 and 16.—Strain N-4, S-25.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The Action of Analogues of DL-Glyceraldehyde on Glycolysis and Respiration of Tumors [1, 2]

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Summary

Three analogues of DL-glyceraldehyde were tested on whole cells of various ascites tumors. Glycidaldehyde and α-methoxyhydracrylaldehyde were found to be potent inhibitors of both glycolysis and respiration, while β-methoxylactaldehyde was only weakly inhibitory. The mechanism of action was presumably through the aldolase reaction in which compounds inhibitory to hexokinase were formed, although glycidaldehyde may readily act as an alkylating agent, while β-methoxylactaldehyde may act to rearrange itself to dihydroxyacetone methyl ether. Glycidaldehyde was more strongly inhibitory to glycolysis than to respiration in the lymphoma only, whereas just the reverse was true in the other tumors. This suggests that lymphomas may be different from other cancers. It is speculated that a specific inhibitor to a specific glycolytic enzyme might provide a practical test of the Warburg theory.—**J. Nat. Cancer Inst. 22: 341–348, 1959.**

Mendel (*1*) reported in 1929 that glyceraldehyde, in concentrations as low as 0.001 M, almost completely inhibits anaerobic glycolysis in cancer cells without affecting the respiratory rate of the cells. He further stated that it is possible that glyceraldehyde prevents the development of cancer cells in the normal organism.

Mendel's observations were substantiated by Baker (*2*) in 1938, and extended to testicular and embryonic tissues, as well as tumor tissues. Baker found that the action of DL-glyceraldehyde was specific, since a similar effect was not obtained with glycolaldehyde, acetaldehyde, propionaldehyde, benzaldehyde, or dihydroxyacetone. Needham and Lehmann (*3*) reported that the inhibition of embryonic glycolysis by DL-glyceraldehyde was entirely due to the L-form; the inhibition was complete at 0.0025 M concentration. The same authors (*4*) found that the inhibi-

[1] Received for publication August 12, 1958.

[2] This investigation was supported by a Postdoctorate Research Fellowship from the National Cancer Institute, National Institutes of Health, Public Health Service.

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[4] The author wishes to thank Dr. D. M. Greenberg for his valuable guidance in the planning of this research and preparation of this paper.

tion by DL-glyceraldehyde appeared to be incomplete because of lactic acid formation from the glyceraldehyde itself. Mendel (*5*) substantiated the fact that, unlike DL-glyceraldehyde, D-glyceraldehyde does not inhibit glycolysis by tumor cells; the inhibition by DL-glyceraldehyde is apparently due to the L-form, the activity of which may be several times as high as that of the DL-form.

Fischer and Baer (*6*) reported in 1936 that a mixture of D-glyceraldehyde and dihydroxyacetone yielded D-fructose and D-sorbose. In that same year Meyerhof *et al.* (*7*) found that crude aldolase preparations condensed dihydroxyacetone phosphate and glyceraldehyde to ketohexose-1-phosphate. With D-glyceraldehyde, D-fructose-1-phosphate is formed, but with racemic aldehyde, a practically equimolar mixture of D-fructose-1-phosphate and L-sorbose-1-phosphate results, the reactions being *irreversible*.

One year later it was demonstrated that, with liver slices, racemic glyceraldehyde is transformed into equimolar quantities of a fermentable hexose and a nonfermentable ketohexose (*8*). Again in 1954 it was reported that rabbit muscle aldolase catalyzes the condensation of both D- and L-glyceraldehyde with dihydroxyacetone phosphate to form D-fructose-1-phosphate and L-sorbose-1-phosphate (*9*).

Süllmann (*10*), working with the lens, suggested that DL-glyceraldehyde prevents esterification of inorganic phosphate. Subsequently a series of papers appeared (*11–14*) showing that glycolysis inhibition by glyceraldehyde was accompanied by an inhibition of phosphate esterification; it was further stressed that while the glycolysis of glucose, fructose, and glycogen in various tissue extracts was inhibited, the glycolysis of hexosediphosphate and hexosemonophosphate was not inhibited. It was inferred that glyceraldehyde interferes with the change of carbohydrate substrate to hexosemonophosphate.

As to the definite mechanism of glycolysis inhibition, Süllmann (*15*) attributed it to the aldolase reaction. Ten years later Rudney (*16*), working with hexokinase extracts of beef brain and acetone powders of rat sarcoma, rat skeletal muscle and yeast, showed these different hexokinase preparations to be inhibited by the L-isomer of DL-glyceraldehyde. Lardy *et al.* (*17*) corroborated the result that various hexokinase preparations were strongly depressed by L-glyceraldehyde but not by D-glyceraldehyde. The evidence suggested that the inhibition was not direct, but that the true inhibitor is formed by condensation of L-glyceraldehyde with dihydroxyacetone phosphate to form L-sorbose-1-phosphate. The latter was prepared synthetically, and was shown to be a potent inhibitor of hexokinase. From this it was inferred that the mechanism of inhibition by glyceraldehyde was through the formation of L-sorbose-1-phosphate, which is the actual inhibitor of the hexokinase reaction.

In 1956 Warburg (*18*) re-emphasized his views on the role of glycolysis in cancer cells; he stressed the hypothesis that over a period of time a cell becomes malignant by virtue of injury to the oxidative respiratory mechanisms and reverts to a more primitive mode of energy-getting,

namely, fermentation. In a recent article, Krebs (*19*) refers to two "pacemakers" of anaerobic glycolysis—the hexokinase reaction and the triosephosphate dehydrogenase reaction.

The guiding consideration in this investigation was that if a substance could be found which was completely inhibitory to glycolysis without affecting respiration it would deprive a malignant cell of most of its energy but would not affect normal cells to a significant degree. With the foregoing in mind, it was decided to reinvestigate the properties of triose inhibitors and their effect upon glycolysis and respiration in cancer cells—presumably their effect upon the hexokinase reaction. The three substances used were: α-methoxyhydracrylaldehyde, β-methoxylactaldehyde (the α- and β-methyl ethers of DL-glyceraldehyde, respectively) and glycidaldehyde (2,3-epoxypropionaldehyde).[5]

Materials and Methods

The α-methyl ether was synthesized according to Hersant and Linnell (*20*). The three compounds used were generously supplied by the Shell Development Company (Emeryville, Calif.). Glycol values indicated a purity of over 99.8 percent in all compounds; theoretical and found values for C, H, and OCH_3 were in agreement. Acetaldehyde and propionaldehyde, which were used as controls, were freshly distilled before use. DL-Glyceraldehyde-3-phosphate was synthesized according to the method of Baer and Fischer (*21*). Glyceraldehyde phosphate dehydrogenase (GAPD) was obtained from Worthington Biochemicals, Inc.

The inhibitory action of the 3 glyceraldehyde analogues on glycolysis and respiration was determined in a Warburg apparatus with the following ascites tumor cells: Ehrlich ascites (ES); Sarcoma 180 (S-180); SX lymphoma (SX); and ED lymphoma (ED). To each Warburg flask was added: buffered saline-phosphate solution, 35 μmoles of glucose, 0.1 ml. of 0.35 M $NaHCO_3$ (for glycolysis). The total volume was 2.4 ml. Respiration was measured by the use of endogenous substrate with 0.2 ml. NaOH in the center well of each flask, the medium contained no bicarbonate. All constituents for glycolysis were added to the main compartment of the flask, with the glucose in the side-arm; the flask, attached to a manometer, was placed in a Warburg bath at 37° C. and equilibrated for 10 minutes with a mixture of 95 percent N_2 and 5 percent CO_2; the stopcocks were closed and another 10 minutes were allowed for further equilibration; the glucose was then tipped into the main compartment and the reaction was started. The respiration runs were done in the same manner, except that the equilibration was done with air and no glucose was added.

[5] The α- and β-methyl ethers of DL-glyceraldehyde phosphate were prepared by phosphorylating the acetals of the α- and β-methyl ethers of DL-glyceraldehyde with diphenylphosphorylchloride (Aldrich Chemical Co.), then removing the diphenyl groups by hydrogenolysis and isolating the respective compounds as the dicyclohexylamine salt. These syntheses, in general, were carried out according to the procedure of Ballou and Fischer (*22*). The resulting compounds, DL-glyceraldehyde-3-phosphate-2-methyl ether and DL-glyceraldehyde-2-phosphate-3-methyl ether, were shown to be only slightly inhibitory to the triose dehydrogenase system when DL-glyceraldehyde-3-phosphate was used as substrate. At a concentration of 0.01 M, these compounds inhibited the formation of DPNH about 20 to 30 percent. They were not used in the whole cell studies.

Assays to test the effectiveness of the analogues, as inhibitors of the triose dehydrogenase system, were done in a Beckman recording spectrophotometer with crystalline GAPD, according to the procedure of Cori, Slein, and Cori (*23*).

Results

The results of the Warburg experiments with whole cells are shown in table 1. The QO_2 represents the amount of oxygen, in $mm.^3$, that 1 mg. of tissue (dry wt.) consumes per hour at 37° C. with oxygen saturation. QMN_2 represents the amount of lactic acid, in $mm.^3$, that 1 mg. of tissue (dry wt.) develops per hour at 37° under anaerobic conditions. If QO_2 is multiplied by 7 and QMN_2 by 1, the number of $mm.^3$ of ATP that 1 mg. of tissue (dry wt.) can synthesize per hour (22,400 $mm.^3$ = 1 mmole ATP) is obtained. These quotients are called $QATPO_2$ and $QATPN_2$, according to whether the ATP is formed by respiration or by anaerobic glycolysis, respectively (*18*).

Tests of the 3 analogues of glyceraldehyde on the triose dehydrogenase system yielded negative results. At a concentration of 0.01 M, none of these substances was inhibitory to the formation of DPNH from DPN.

TABLE 1.—*Inhibition of glycolysis and respiration by glyceraldehyde analogues*

With glycidaldehyde

7-Day S-180 ascites

Molar concentration	Respiration: Inhibition (percent)	Respiration: QO_2	Respiration: $QATPO_2$	Glycolysis: Inhibition (percent)	Glycolysis: QMN_2	Glycolysis: $QATPN_2$	$QATP^N{}_2/_{O2}$
Control	—	28. 6	200. 2	—	80. 2	80. 2	0. 40
1×10^{-2}	90	2. 8	19. 6	82	14. 8	14. 8	0. 75
7×10^{-3}	91	2. 5	17. 5	84	13. 2	13. 2	0. 75
4×10^{-3}	87	3. 8	26. 6	55	36. 4	36. 4	1. 37
1×10^{-3}	51	14. 1	98. 7	28	57. 7	57. 7	0. 58
5×10^{-4}	31	19. 6	137. 2	25	60. 7	60. 7	0. 44

7-Day Ehrlich ascites

Control	—	18. 3	128. 1	—	62. 4	62. 4	0. 49
1×10^{-2}	94	1. 1	7. 7	85	9. 3	9. 3	1. 21
7×10^{-3}	91	1. 5	10. 5	84	9. 4	9. 4	0. 90
4×10^{-3}	89	2. 0	14. 0	71	17. 6	17. 6	1. 26
1×10^{-3}	61	7. 1	49. 7	28	43. 8	43. 8	0. 88
5×10^{-4}	25	13. 7	95. 9	3	59. 2	59. 2	0. 62

7-Day SX lymphoma

Control	—	7. 2	50. 4	—	25. 2	25. 2	0. 50
1×10^{-2}	79	1. 5	10. 5	98	0. 6	0. 6	0. 06
7×10^{-3}	70	2. 2	15. 4	93	1. 8	1. 8	0. 12
4×10^{-3}	52	3. 4	23. 8	96	1. 0	1. 0	0. 04
1×10^{-3}	5	6. 8	47. 6	35	16. 3	16. 3	0. 34
5×10^{-4}	—	8. 1	56. 7	14	21. 7	21. 7	0. 38

TABLE 1.—*Inhibition of glycolysis and respiration by glyceraldehyde analogues*—Con.

With β-methyl ether of DL-glyceraldehyde

7-Day S-180 ascites

Molar concentration	Respiration: Inhibition (percent)	Respiration: QO_2	Respiration: $QATPO_2$	Glycolysis: Inhibition (percent)	Glycolysis: QMN_2	Glycolysis: $QATPN_2$	Glycolysis: $QATPN_2/O_2$
Control	—	18. 6	130. 2	—	79. 7	79. 7	0. 61
1×10^{-2}	78	4. 2	29. 4	54	36. 9	36. 9	1. 26
7×10^{-3}	66	6. 3	44. 1	48	41. 2	41. 2	0. 93
4×10^{-3}	59	7. 8	54. 6	37	50. 0	50. 0	0. 92
1×10^{-3}	6	17. 3	121. 1	5	76. 6	76. 6	0. 63
5×10^{-4}	—	20. 3	142. 1	11	70. 5	70. 5	0. 50

7-Day Ehrlich ascites

Control	—	22. 9	160. 3	—	82. 8	82. 8	0. 52
1×10^{-2}	61	8. 8	61. 6	30	58. 1	58. 1	0. 94
7×10^{-3}	49	11. 7	81. 9	23	64. 0	64. 0	0. 78
4×10^{-3}	27	16. 7	116. 9	12	73. 0	73. 0	0. 62
1×10^{-3}	—	23. 9	167. 3	8	77. 0	77. 0	0. 42

7-Day SX lymphoma

Control	—	10. 5	73. 5	—	40. 9	40. 9	0. 56
1×10^{-2}	38	6. 4	44. 8	39	25. 2	25. 2	0. 56
7×10^{-3}	29	7. 5	52. 5	30	28. 6	28. 6	0. 54
4×10^{-3}	14	9. 2	64. 4	22	32. 0	32. 0	0. 50
1×10^{-3}	17	8. 8	61. 6	37	27. 4	27. 4	0. 44
5×10^{-4}	10	9. 6	67. 2	5	38. 7	38. 7	0. 58

With α-methyl ether of DL-glyceraldehyde

7-Day S-180 ascites

Control	—	19. 1	134. 5	—	73. 7	73. 7	0. 35
1×10^{-2}	71	5. 5	38. 7	89	8. 2	8. 2	0. 21
7×10^{-3}	50	9. 5	66. 6	84	11. 6	11. 6	0. 17
5×10^{-3}	35	12. 4	86. 7	72	20. 9	20. 9	0. 24
3×10^{-3}	7	17. 9	125. 1	51	36. 3	36. 3	0. 29
1×10^{-3}	7	17. 9	125. 4	13	64. 6	64. 6	0. 52
5×10^{-4}	—	19. 1	134. 4	—	75. 7	75. 7	0. 57
1×10^{-4}	—	18. 9	132. 5	—	71. 9	71. 9	0. 54

7-Day Ehrlich ascites

Control	—	18. 0	125. 8	—	58. 5	58. 5	0. 47
1×10^{-2}	61	7. 1	49. 5	76	14. 2	14. 2	0. 29
7×10^{-3}	51	8. 8	61. 3	73	15. 8	15. 8	0. 26
5×10^{-3}	49	9. 2	64. 1	68	18. 9	18. 9	0. 29
3×10^{-3}	22	14. 1	98. 8	62	22. 3	22. 3	0. 22
1×10^{-3}	34	12. 0	83. 7	51	28. 7	28. 7	0. 34
5×10^{-4}	18	14. 8	103. 9	17	48. 9	48. 9	0. 47
1×10^{-4}	10	16. 1	113. 0	—	59. 1	59. 1	0. 52

TABLE 1.—*Inhibition of glycolysis and respiration by glyceraldehyde analogues*—Con.

With α-methyl ether of DL-glyceraldehyde—Continued

7-Day SX lymphoma

Molar concentration	Respiration: Inhibition (percent)	Respiration: QO_2	Respiration: $QATPO_2$	Glycolysis: Inhibition (percent)	Glycolysis: QMN_2	Glycolysis: $QATPN_2$	Glycolysis: $QATP^N{}_2/O_2$
Control	—	11. 4	80. 0	—	48. 3	48. 3	0. 60
1×10^{-2}	49	5. 8	40. 8	74	12. 4	12. 4	0. 30
7×10^{-3}	29	8. 1	56. 8	78	10. 6	10. 6	0. 19
5×10^{-3}	18	9. 4	65. 7	47	25. 5	25. 5	0. 39
3×10^{-3}	21	9. 0	63. 3	22	37. 6	37. 6	0. 59
1×10^{-3}	16	9. 6	67. 4	28	34. 9	34. 9	0. 52
5×10^{-4}	16	9. 7	67. 7	4	46. 5	46. 5	0. 69
1×10^{-4}	12	10. 1	70. 6	12	42. 6	42. 6	0. 60

7-Day ED lymphoma

Control	—	12. 7	88. 8	—	50. 1	50. 1	0. 56
1×10^{-2}	53	6. 0	41. 7	85	7. 8	7. 8	0. 19
7×10^{-3}	54	5. 9	41. 0	89	5. 6	5. 6	0. 14
5×10^{-3}	57	5. 5	38. 3	85	7. 7	7. 7	0. 20
3×10^{-3}	42	7. 3	51. 3	29	35. 9	35. 9	0. 70
1×10^{-3}	50	6. 3	44. 1	19	41. 4	41. 4	0. 94
5×10^{-4}	36	8. 1	56. 6	19	41. 0	41. 0	0. 72
1×10^{-4}	38	7. 9	55. 4	—	51. 1	51. 1	0. 92

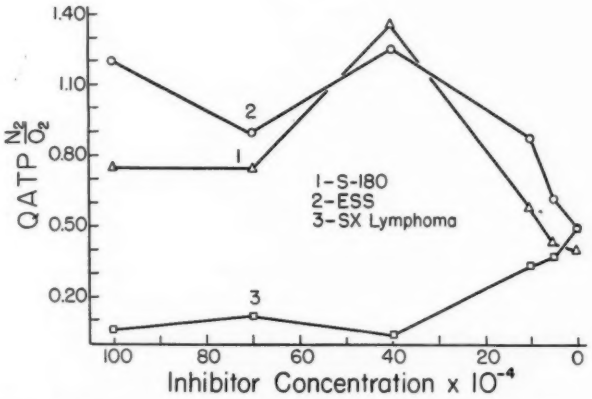
All 3 analogues were inhibitory to both glycolysis and respiration in the Warburg determinations. Concentrations of 0.01 M inhibited both processes 30 to 95 percent, while concentrations of 0.0005 M inhibited 0 to 25 percent. The α-methyl ether was more inhibitory to glycolysis than to respiration in all tumors. The β-methyl ether was a much weaker inhibitor than the other two, and inhibited respiration more than glycolysis. Glycidaldehyde was a potent inhibitor and had differential effects on the tumors tested.

Discussion

Since none of the compounds affected the triose dehydrogenase system, the mechanism of action appeared to be at the hexokinase level through the aldolase reaction, as with L-glyceraldehyde. However, there may very well be other mechanisms. The β-methyl ether may rearrange into dihydroxyacetone methyl ether (*6*) and thus inhibit by competition with dihydroxyacetone or through the aldolase reaction, forming hexose(s) which may compete with glucose.

In addition, glycidaldehyde may be a potent alkylating agent by virtue of its unstable epoxy ring. With this compound there is a distinct difference in action upon the lymphoma in contrast with the other tumors. In the lymphoma, glycolysis is inhibited to a greater extent than respiration; in the other tumors just the reverse is true. This is depicted in

text-figure 1, where $QATPN_2/O_2$ represents the ratio of energy produced by fermentation against that produced by respiration. Small quotients represent better inhibitors of glycolysis, whereas higher quotients represent better inhibitors of respiration, relatively speaking; the differential response between the lymphoma and other tumors appears significant. In our laboratory other alkylating agents seem to have this same differential effect on lymphomas, but not so in other tumors. This may be further evidence pointing to a distinct difference between lymphomas and other cancers.



TEXT-FIGURE 1.—Differential inhibition of glycolysis and respiration by glycidaldehyde.

The original incentive for this research was to develop an agent that would strongly inhibit glycolysis but have little or no effect on respiration. In such a way it was thought that the apparent difference in energy-getting mechanisms between cancer and normal cells might best be exploited. A strong glycolytic inhibitor might cause those cancer cells which depended mainly on glycolysis to die, while making only a small indentation upon a normal cell's energy. To this end it was speculated that fairly specific inhibitors to the glycolytic enzymes alone, should be devised; specifically, that an irreversible inhibitor to the "pacemakers" of glycolysis (*19*) is needed, *i.e.*, to either hexokinase or glyceraldehyde phosphate dehydrogenase. It is now felt that inhibition of the latter may be more fruitful than that of the former. But, in this respect the α- and β-methyl ethers of glyceraldehyde phosphate (*see* footnote 5) had little effect upon the enzyme itself. However, a compound such as α-mercaptoglyceraldehyde-3-phosphate, if developed, might act not only as a competitive inhibitor of the genuine triose phosphate but also, through its mercapto group, might irreversibly bind the enzyme by formation of a disulfide linkage with one of the sulfhydryl groups on the enzyme itself. Such a compound, if active, might be an agent by which the Warburg theory could be tested.

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Synthesis, Pharmacology, and Clinical Trial of the Riboflavine Analogue, Sodium-6,7-Dimethyl-9-(2′-Hemisuccinoxyethyl)-Isoalloxazine, U-6538 [1]

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Summary

The synthesis of a riboflavine analogue, sodium-6,7-dimethyl-9-(2′-hemisuccinoxyethyl)-isoalloxazine, U-6538, is reported. Administered parenterally this compound produced inhibition of rat growth which could be reversed by riboflavine. The drug was capable of inhibiting the growth of a rat lymphosarcoma. Prolonged parenteral administration of U-6538 was well tolerated by rats and dogs. A trial of U-6538 was conducted in 4 patients with malignant neoplasms. Drug dosage was limited by precipitation of a metabolite in the urinary tract. Some patients had signs of renal injury. No definite evidence was obtained of clinical riboflavine deficiency or antitumor effect. An explanation for this is based on the metabolism of the drug.—**J. Nat. Cancer Inst. 22: 346–361, 1959.**

It has been noted that as nutrition of the terminally ill cancer patient becomes poor the rate of tumor growth may decrease. Nutritional restrictions have been shown to depress tumor formation (*1–3*) and growth (*4–11*) in experimental animals. The induction of riboflavine deficiency through the use of riboflavine-deficient diets and riboflavine antagonists has resulted in tumor regression in mice and rats (*12–17*). It is conceivable that tumor regression in man could be induced by riboflavine deficiency. Dietary restriction of this vitamin alone leads to signs of deficiency only after several months and in a small proportion of patients (*18*). This approach seems impractical for therapeutic evaluation in cancer patients. Several riboflavine antagonists have been tested in man (*19*, *20*). The riboflavine analogue 6,7-dimethyl-9-(2′-acetoxyethyl)-isoalloxazine, U-2112, is an active riboflavine antagonist for bacteria and rodents. Its pharmacology and clinical trial have recently been reported by Lane *et al.* (*20*). Clinical riboflavine deficiency could not be induced in patients

[1] Received for publication August 12, 1958.

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[4] The authors wish to thank Drs. Harry H. Fall and Richard L. Johnston for their help in obtaining some of these data and Mrs. Frances L. Francis for technical assistance.

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given the compound orally. Parenteral administration was obviated by the low solubility of the analogue in aqueous solution.

In order to obtain a more soluble compound with activity as a riboflavine antagonist, the sodium hemisuccinate of 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine, U-2113, was synthesized by one of us (H. G. P.). This is sodium-6,7-dimethyl-9-(2′-hemisuccinoxyethyl)-isoalloxazine, or U-6538. It is very soluble in water and only slightly soluble in acid. Its pharmacology in bacteria and animals was studied and antitumor activity was observed in rats. Dogs tolerated well intravenous injections of the drug. In view of the great solubility and the low animal toxicity of U-6538, as well as its ability to induce regression of rat tumors, the compound was selected for trial in man in an attempt to induce riboflavine deficiency and/or tumor regression. The synthesis of U-6538, bacteriological and animal studies, and pharmacological trial in 4 patients with neoplastic diseases are reported.

Methods

Preparation of U-6538.—By a method similar to that described by Fall and Petering (*21*) for the preparation of 6,7-dimethyl-9-(2′-acetoxyethyl)-isoalloxazine, 6,7-dimethyl-9-(2′-hemisuccinoxyethyl)-isoalloxazine was prepared. From this compound the sodium salt was obtained as a buffered preparation.

a) 6,7-Dimethyl-9-(2′-hemisuccinoxyethyl)-isoalloxazine (*I*).—One hundred gm. (0.35 mole) of 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine was refluxed with 100 gm. (1.0 mole) of succinic anhydride in 1500 ml. of dry pyridine for 1½ hours. The mixture was filtered hot and insoluble matter discarded. The volume of the filtrate was reduced at atmospheric pressure to about 500 ml., at which concentration a solid precipitated. The mixture was allowed to cool to room temperature and the solid was removed by filtration. The precipitate was washed twice with 100 ml. of glacial acetic acid to remove pyridine and then with ether to remove the glacial acetic acid. The solid was dried at 80° C. overnight. The yield was 119 gm. (89% of theory); m.p. 229.5 to 230° (Fisher block); UV maximum 270 mμ/355 mμ—0.940/0.310 = 3.03 (10 mg./liter in 0.1 N NaOH). $\frac{aM(270\ m\mu)}{aM(355\ m\mu)} = \frac{36{,}300}{11{,}970} = 3.03$. A similar sample was purified by heating 2.0 gm. with 50 ml. of dry pyridine to boiling and filtering the solution free of a small amount of residue. The pyridine solution was treated with an equal volume of dry ether and the precipitate was collected, washed with 50 percent aqueous acetic acid, acetone, and ether.

Analysis: $C_{18}H_{18}N_4O_6$. C 56.0 percent, H 4.7 percent, N 14.5 percent, m.p. 227 to 228°.

Found: C 55.96 percent, H 4.56 percent, N 14.19 percent, pk_a 5.2, 10.6 (60% ethanol).

b) Sodium-6,7-dimethyl-9-(2′-hemisuccinoxyethyl)-isoalloxazine (*U-6538*) (*II*).—Six hundred gm. (1.55 moles) of (I), not recrystallized, was suspended in 3.5 liters of water. To this mixture was added 980 ml. of 1.425 molar solution of sodium hydroxide (AR) with constant stirring. The *p*H

at the end of the procedure was 7.6. There was a faint odor of pyridine present, which was removed during the subsequent procedures. The mixture was filtered through celite to remove insoluble material. This material was found to be unreacted (I) resulting from the use of less than an equivalent amount of alkali. (Calculations indicate 98% neutralization of soluble material.) It amounted to 50 gm. (0.13 mole). The filtrate had satisfactory analytical constants that are described. After dilution to 5.8 liters, it was filtered aseptically through a Selas filter (02 porosity) and the effluent was used to fill vials for clinical use. The vials, which contained 1.047 gm. of solid, were then subjected to freeze drying. The solution for intravenous or intramuscular administration was obtained by adding sterile water to the vial. The solid is stable for months if shielded from light. Analytical data of (II): 1) Solubility—20 percent weight per volume; 2) *p*H of reconstituted vials, 7.5; 3) R_F—0.75, *n*-butanol, water, acetic acid (7:2:1), Whatman # 4 paper; 4) ultraviolet spectra—a) in water $\frac{aM(265\ m\mu)}{aM(370\ m\mu)} = \frac{30,600}{10,250} = 2.99$ (concentration, 10 mg./liter), b) in 0.1 N NaOH $\frac{aM(270\ m\mu)}{aM(355\ m\mu)} = \frac{34,200}{11,550} = 2.98$ (concentration, 10 mg./liter); 5) fluorescence—similar to riboflavine.

Animal toxicity: a) Rodents.—The LD50 of U-6538 by the intraperitoneal route was determined in Swiss mice. Sprague-Dawley rats weighing approximately 120 gm. and bearing 1-day-old subcutaneous implants of the Murphy-Strum lymphosarcoma received various doses of U-6538 intraperitoneally daily for up to 3 weeks. Ten rats were treated at each dose level.

b) Dogs.—Acute and chronic toxicity studies were performed in dogs weighing 7 to 13 kg. U-6538 was injected as an aqueous solution intravenously. Blood and urine samples were taken prior to the study and at various intervals for examination. Hemoglobin, hematocrit, leukocyte count, differential count, and complete urinalysis were performed.

Growth inhibitory experiments: a) Bacteria.—The turbidity resulting from growth of *Lactobacillus casei*, A.T.C.C. #7469, was used to assay riboflavine-like and antiriboflavine activity, and was determined at 600 mμ with the Beckman B spectrophotometer. In early experiments tubes containing bioassay medium were autoclaved after addition of U-6538. In later studies autoclaving was performed before addition of U-6538.

b) Rats.—Male Wistar weanling rats were divided into 4 groups of 10 animals per group. These animals received a purified diet containing 18 percent casein and no riboflavine, but otherwise complete in known growth factors. When the weight of the animals in group 1 plateaued (8th day), they were injected intraperitoneally with 200 μg. of riboflavine per kg. of body weight until the 15th day, at which time the riboflavine dose was increased to 400 μg. per kg. Groups 2 and 3 were injected daily with 200 μg. of riboflavine per kg. of body weight and 400 μg. per kg., respectively, from the start. Group 4 was injected intraperitoneally with U-6538 at 10 mg. per kg. for 7 days until it was definitely established that

the drug caused the animals to lose weight precipitously, at which time (8th day), in addition to this drug, riboflavine was given at 200 μg. per kg. for 6 days and then increased to 400 μg. per kg.

Antitumor studies in rats.—Sprague-Dawley male weanling rats were fed a purified, complete diet containing 25 percent casein and riboflavine at 8 mg. per kg. of feed. When the animals were about 120 gm. in weight, cell suspensions of Murphy-Sturm lymphosarcoma were inoculated subcutaneously into the axillary region. Each rat was given 2×10^6 cells. U-6538 was injected intraperitoneally beginning on the 1st day after implantation. The doses and schedules are tabulated in the results.

Chemical studies.—The method for determination of plasma total flavine concentrations was the same as reported by Lane *et al.* (*20*). In the present studies the sodium hydrosulfite reducible fluorescence of neutralized supernatants from trichloroacetic acid-precipitated plasma was compared with similarly treated U-6538 standards. Total flavine concentrations of the urine of patients on the drug were determined by the fluorescence of urine diluted from 1:100 to 1:2000 with distilled water, since other sources of fluorescence were insignificant in comparison due to the large quantities of flavine excreted after administration of U-6538. The dilution chosen for each sample was such that the final flavine concentration of the diluted urine would be from 0.1 to 2.0 μg. per ml., in which range the relationship between fluorescence and flavine concentration was found to be linear. The Coleman photofluorometer and appropriate filters for flavines (Corning #3389, 5113, and 3486) were employed for the fluorescence measurements. Urine-flavine metabolites were separated by descending paper chromatography on Whatman #3MM paper, with butanol:acetic acid:water (4:1:5) or butanol:pyridine:water (6:4:3) as the developing solvents, and were eluted and quantified as previously reported (*20*).

The yellow crystals obtained in the urine of some patients receiving U-6538 were separated by centrifugation and washed by repeated suspension in small volumes of water and then ether. These crystals were slightly soluble in water and formed a yellow solution which was intensely fluorescent, resisted mild oxidation by potassium permanganate, and was easily reduced by sodium hydrosulfite. The fluorescence spectrum in neutral aqueous solutions was identical with that previously described for several flavines. On paper chromatography a solution of the crystals gave a single spot which corresponded in mobility to U-2113.

Clinical studies.—Four patients who had histologically verified disseminated neoplasms participated in this study. They received diets calculated to contain 0.5 mg. riboflavine per day. Oral supplements of other vitamins were administered daily: vitamin A—5000 U., vitamin D—1000 U., thiamin hydrochloride—5 mg., pyridoxine hydrochloride—5 mg., nicotinic acid—5 mg., folic acid—5 mg., and ascorbic acid—75 mg. An attempt was made to provide an intake of 30 to 50 gm. protein and over 1,000 calories a day, but this was difficult to maintain in all patients. Hematopoietic, hepatic, and renal functions were studied by the usual

clinical laboratory tests. The drug was first administered intravenously in 5 percent dextrose solution over periods of 1 to 4 hours daily in increasing doses until signs of toxicity appeared. Plasma total flavine levels were determined at 1- to 8-hour intervals for 24 hours. Urine total flavine excretion was determined in 24-hour collections. The same was done for the intramuscular route of administration. After these maximum-tolerated doses were determined, patients were maintained on lower doses injected intramuscularly during the trial period. Daily examinations were made for signs and symptoms of riboflavine deficiency, especially glossitis, cheilosis, seborrheic or other dermatitis, corneal vascularization, and drug toxicity, especially urinary-tract dysfunction and crystalluria. Antitumor effect was assayed by the direct measurement of metastatic lesion size and number once weekly for the cutaneous and subcutaneous lesions, and by the measurement of pulmonary lesions demonstrated on roentgenograms taken in a standard manner every 2 weeks (*22*).

Results

Animal Toxicity

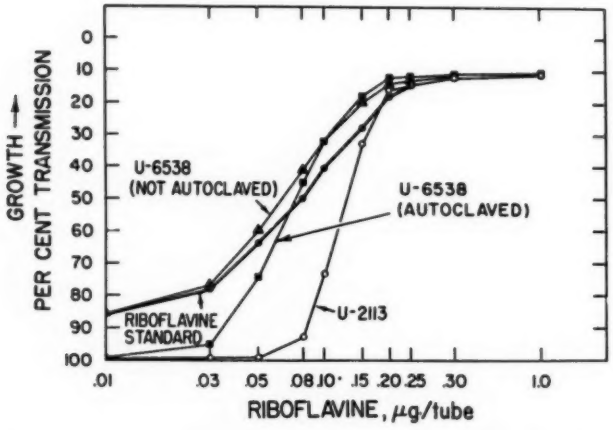
a) *Rodents.*—The acute LD50 in Swiss mice after intraperitoneal injection was 767 mg. per kg. Daily injection of 20 mg. per kg. was well tolerated for 3 weeks by Sprague-Dawley rats bearing the Murphy-Sturm tumor. At doses of 60 mg. per kg. deaths were noted after 5 days. The only significant pathological finding was a deposition of drug or drug metabolite in the uriniferous tubules of the rats. In some instances renal obstruction was probably the cause of death.

b) *Dogs.*—Single intravenous doses of U–6538 of 75 and 150 mg. per kg. of body weight were accompanied by yellow discoloration of mucous membranes, licking of lips, salivation, retching, defecation, and irregularity of the pulse. The dogs returned to normal within 10 minutes. At the 500 mg. per kg. level the dog appeared depressed for about 1½ hours. No overt ill effects following daily injections of 50 and 100 mg. per kg. 5 days a week for 4 weeks were noted, except for the acute reaction, previously mentioned, which followed each injection. Weight loss was negligible. No significant changes occurred in hemoglobin concentration, hematocrit, white blood cell count, or differential count. Urine examinations were unremarkable except for the occasional presence of an orange-brown precipitate. The dogs were killed and autopsied 4 days after the last injection. The significant findings were the presence of fairly large amounts of amorphous yellow material in the gall bladders of some animals and occasional small, yellow deposits in the kidney tubules without evidence of renal damage.

Growth Inhibition

a) *Bacteria.*—U-6538 had no activity as a riboflavine antagonist for the growth of *L. casei* at a 5000:1 ratio of drug to riboflavine, when the drug was added to the tubes containing culture media after they had been

autoclaved and cooled. Chromatograms prepared from these cultures after 24 hours of incubation demonstrated the major flavine to be unchanged U-6538. When bioassay was performed on media autoclaved after the addition of U-6538, then cooled, and inoculated with *L. casei*, bacterial growth was inhibited at a ratio of initial drug to riboflavine of 1600:1 (text-fig. 1). Chromatograms of this material demonstrated 2 spots of about equal intensity, corresponding to U-6538 and to U-2113. U-2113 is an inhibitor of *L. casei* at a ratio of 500:1. These findings indicate that autoclaving U-6538 in bioassay medium results in its hydrolysis to U-2113, with an attendant increase in inhibition of bacterial growth due to the presence of this more active riboflavine antagonist. This suggests that in the rat and in man the activity of U-6538 would be related to the amount of drug present in hydrolyzed form (U-2113).

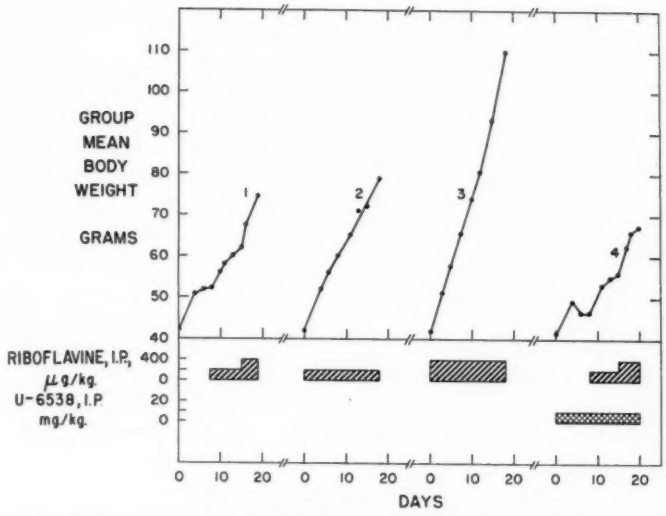


TEXT-FIGURE 1.—Increased activity of U-6538 as a riboflavine antagonist for the growth of *L. casei* after the drug had been autoclaved in bioassay medium. Drug riboflavine inhibition ratios: U-2113 = 500:1; U-6538 (not autoclaved) = no inhibition; U-6538 (autoclaved) = 1666:1; U-6538 and U-2113 concentrations are 50 μg. per tube (10 ml.).

b) *Rats.*—Text-figure 2 indicates that U-6538 administered parenterally resulted in inhibition of rat growth and that this effect could be reversed with riboflavine. The data available give no quantitative information of its potency. In other experiments it has been determined that U-6538 given orally at much higher levels has little or no antiriboflavine activity, which probably results from the fact that it is very poorly absorbed from the intestinal tract.

Antitumor Activity

The data in table 1 indicate that U-6538 is an effective antitumor agent for the Murphy-Sturm lymphosarcoma.



TEXT-FIGURE 2.—Inhibition of growth of weanling Wistar male rats injected intraperitoneally with U-6538. Ten rats per group. Diet devoid of riboflavine.

TABLE 1.—*Effect of U-6538 on growth of the Murphy-Sturm lymphosarcoma in Sprague-Dawley rats*

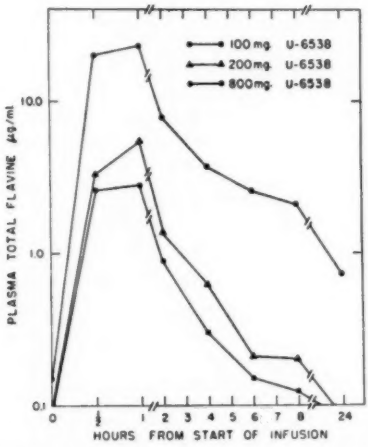
Group	Dose, intraperitoneal (*mg./kg.*)	Average weight change (*gm.*)—26-day period	Survivors	Average tumor diameter on day 26 (*mm.*)	Regression on day 26 (*rats without palpable tumors*)	Percent inhibition
Control	—	126	8/10	56	1	0
U-6538	2.5, daily	117	7/10	67	0	0
U-6538	5.0, daily	122	7/10	19	5	66
U-6538	5.0, 3× week	139	3/10	63	0	0
U-6538	10.0, 3× week	133	9/10	27	5	52

Clinical Studies

Four patients received trial doses of U-6538. Their case histories are appended to this report. During administration of a single dose of the drug by the intravenous route over a period of 1 hour, the plasma total flavine concentration rose rapidly and was maximal at the completion of the infusion. These levels diminished gradually over an 8-hour period and usually returned to pretreatment baselines at 24 hours. The plasma concentrations increased arithmetically with increments in dosage, as shown for one patient in text-figure 3. Similar families of curves were obtained with corresponding intramuscular doses. However, the maximum levels, which occurred in ½ to 1 hour, were somewhat lower and the 8-hour levels tended to be higher. After a single dose, most of the drug was excreted in the urine in the first 8 hours and for small doses 90 to 100 percent was recovered in 24 hours. With doses of 600 and 800

mg., the percentage of drug recovered in the urine at 24 hours fell as low as 67 percent.

Patients with plasma flavine concentrations approximating 10 μg. per ml. usually had flank pain, oliguria, and crystalluria. The crystals were shown to be a drug metabolite, U-2113. If drug administration was discontinued and fluid intake was maintained at 2 to 3 liters per day, these findings abated in 18 hours. The drug was best tolerated when the urine output was over 2 liters per day. Doses that produced crystalluria on intramuscular injection could occasionally be administered as a 4-hour infusion without complication. In one patient, when the urine output was low, crystalluria followed a dose as small as 50 mg. (1 mg./kg.) intramuscularly. If a tolerated-intramuscular dose was repeated at 8-hour intervals, crystalluria sometimes occurred. A moderate reduction of the single tolerated dose usually permitted repetitive administration at 8-hour



TEXT-FIGURE 3.—Plasma total flavine concentrations for a patient receiving increasing single intravenous doses of U-6538. The doses were given as 1-hour infusions.

intervals without incident. Daily schedules for maintenance therapy were 150 to 800 mg. (3 to 12 mg./kg.) in divided doses every 6 to 8 hours intramuscularly. Total doses ranged from 6.9 gm. in 47 days to 53 gm. in 117 days. One patient could not tolerate 150 mg. (3 mg./kg.) daily when given intramuscularly because of renal toxicity, while another tolerated 800 mg. (12 mg./kg.) daily by the same route. In one instance, a patient developed edema and oliguria after having the drug for about 3 months. Another patient developed albuminuria and edema after 2 months of drug trial. At *post-mortem* examination the latter patient had some necrosis of renal-tubular epithelium and deposits of a yellow-brown pigment in the renal tubules, the Kupffer cells of the liver, and the phagocytic cells of the adrenals. This patient had very widely disseminated melanoma and had melanuria; these deposits may have been

melanin. None of the other patients had such changes at *post-mortem* examination. No other evidences of significant toxicity were observed. No disturbances of the hematopoietic, cardiac, or central-nervous system functions were found. An episode of jaundice which occurred in one patient could not definitely be ascribed to U-6538 administration, since the patient had received blood transfusions and chlorpromazine and he expired on the day that icterus was first noted.

Changes suggestive of riboflavine deficiency occurred in 2 patients. However, in both instances the clinical picture was complicated by other factors which made the diagnosis of isolated riboflavine deficiency uncertain. One patient received the drug when terminally ill with melanoma, which was metastatic throughout the central-nervous system and the gastrointestinal tract. She had continued nausea and vomiting, low urine output, and her nutritional status was poor. She developed cheilosis, glossitis, and an erythematous cutaneous eruption, but expired before an attempt could be made to reverse these changes with supplemental riboflavine. Another patient developed erythema of the nose and fingers, cheilosis, glossitis, stomatitis, dysphagia, and increased dryness of her skin. These occurred only after she received 6-mercaptopurine for 10 days in addition to the U-6538 and the riboflavine-deficient diet; such signs had not developed on the diet and U-6538 alone in 97 days. These signs and symptoms abated when 6-mercaptopurine was discontinued, though she remained on the diet and U-6538. The toxic effect of 6-mercaptopurine may have been accentuated here. No antitumor effect was noted in any of the 4 patients.

Five flavines were obtained from human urine by chromatography. One had an R_F identical with U-6538 and another had an R_F identical with U-2113 in 2 solvent systems. The other 3 fractions had R_F's which were smaller than that of U-2113 and were not the same as for riboflavine. These were designated fractions A, B, C. Fractions having the same R_F values were found in the urine of patients who had received U-2112 and U-2113 (*20*). Fractions A, B, and C were not riboflavine antagonists for *L. casei*. The relative abundance of these fractions in the urine of each patient is shown in table 2. Note that all patients excreted a large

TABLE 2.—*Flavine composition of urine of patients given intramuscular injections of U-6538, as determined following paper chromatography*

Patient	Flavine fraction, percent total fluorescence*				
	U-6538	U-2113	Fraction A	Fraction B	Fraction C
1	36.4	37.3	10.2	8.1	8.0
2	56.2	31.8	5.4	3.0	3.6
3	35.5	41.8	4.6	11.2	6.9
4	43.9	40.6	7.7	3.0	4.8
Mean.....	43.0	37.8	7.0	6.3	5.8

*Each value represents the average of 2 determinations.

proportion in the unchanged U-6538 form. Patient #2 tolerated the largest doses and he excreted the largest proportion in unchanged form. Most of the patients excreted only 30 to 40 percent in the more active U-2113 form.

Discussion

Of the various 9-substituted flavine derivatives studied in this laboratory, the acetoxyethyl flavine, U-2112, is the most active in bacterial systems—being 10 times as active as ethanol flavine (U-2113). As stated previously, no deficiency could be obtained with U-2112 in human beings. Various reasons for this have been presented *(20)*. U-6538, the soluble derivative of U-2113, is not a riboflavine antagonist in bacterial systems. In man it is hydrolyzed to a certain extent to the moderately active ethanol flavine, U-2113. However, neither U-2113 nor any of the other metabolic products of U-6538 are nearly as active as U-2112. Also, when an attempt was made to administer large doses of U-6538, toxicity occurred in the form of crystallization of a drug metabolite (U-2113) in the urinary tract or the patients excreted a larger portion of the drug in the unchanged inactive form. At maximally tolerated doses no definite riboflavine deficiency was induced in 4 patients in from 1 to 3 months. One patient may have developed a nutritional deficiency but this was probably a multiple deficiency which occurred when all vitamins and protein were not being taken in adequate quantities. The drug has definite urinary-tract toxicity. Two patients became edematous. Progressive impairment of renal function occurred in 1 patient and albuminuria in another. These changes were probably secondary to prior episodes of acute renal toxicity when crystals of the relatively insoluble U-2113 were precipitated in the urinary tract.

The only active riboflavine antagonist among the metabolic products of U-6538 was U-2113. It could be expected that the activity of U-6538 in human beings would be dependent on the dose administered as well as the extent of its conversion to U-2113. When patients received U-2112, they excreted U-2112 and U-2113 as the active metabolic products. They tolerated about 100 mg. per kg. per day orally and excreted about 4 percent of this in the urine. Fifty percent of urine flavine was U-2113 and 1 to 2 percent was U-2112. This is the equivalent of 3 mg. per kg. per day of flavine of the activity of U-2113. The patient who tolerated the largest dose of U-6538 received 12 mg. per kg. per day. He excreted 31 percent of this as U-2113 and the remainder as inactive fractions. This is equivalent to 4 mg. per kg. per day of flavine of the activity of U-2113. Thus, the maximum-tolerated doses of U-2112 and U-6538 resulted in essentially the same amount of active riboflavine antagonist being excreted in the urine. Ten patients were given U-2112 without clinical riboflavine deficiency having been induced. Since U-6538 gave little more total active antagonist at the maximum-tolerated dose, it is unlikely to be more effective clinically than U-2112.

It is interesting that the toxicity of 6-mercaptopurine occurred so quickly when combined with the riboflavine-deficient diet and U-6538. This

may mean that the antitumor activity of 6-mercaptopurine might also be potentiated. Investigation of this possibility is currently in progress.

Case Reports

Patient #1.—A 63-year-old white female developed pleuritic pain, pleural effusion, and axillary adenopathy 8 months before admission. Biopsy of an axillary lymph node showed adenocarcinoma. On admission she was emaciated. There were enlarged cervical and axillary lymph nodes. Cutaneous and subcutaneous nodules were present over the anterior chest and in the right breast. She had hepatomegaly and a right pleural effusion. Laboratory tests were normal. An EKG showed low voltage. She received 100 mg. of U-6538 intramuscularly every 12 hours for 5 days, then 100 mg. every 8 hours for 15 days, and then 150 mg. every 8 hours for 77 days. No definite drug toxicity, antitumor effect, or evidence of riboflavine deficiency was seen. She then received, in addition, 6-mercaptopurine at a dose of 50 mg. every 12 hours for 10 days, when she developed angular stomatitis, glossitis, erythema of the nose and fingers, and some scaling of the skin behind the ears. The platelet count fell from 243,000 to 56,000 per mm.^{3}, and the 6-mercaptopurine was discontinued. One week later the leukocyte count had fallen to 425 cells per mm.^{3} Ten days later she became edematous, had oliguria, and a serum sodium of 114 mEq. per liter. The U-6538 was stopped. The riboflavine deficient diet was continued for an additional 10 days, during which time the oral lesions and erythema of the fingers cleared. She had received 53 gm. of U-6538 in 117 days. During the study the serum albumin fell from 3.6 to 2.2 gm. per 100 ml. Inferior vena-caval obstruction developed which was temporarily relieved by treatment with 3000 r of X radiation to the abdomen. When the U-6538 trial ended, the patient remained edematous, developed a precordial friction rub, increased pleural effusions, and she expired 6 weeks later. On *post-mortem* examination the primary tumor was found in the ovary with widespread metastases.

Patient #2.—A 54-year-old white male had a fibrosarcoma of the nasopharynx which had recurred after multiple surgical excisions and 9000 r of X radiation. On admission he had a large fungating tumor in the right nasopharynx and maxillary sinus which extended into the floor of the orbit. It was partially excised but recurred locally within a few weeks. It bled frequently and the patient required numerous blood transfusions. When the trial of U-6538 was started, the laboratory studies were normal. He was given 200 mg. of U-6538 intramuscularly every 6 hours for 32 days without evident drug toxicity, antitumor effect, or riboflavine deficiency. Concomitant 6-mercaptopurine administration, 125 mg. per day orally, was then begun. He developed a septicemia caused by *Pseudomonas aeruginosa* that subsided with antibiotic therapy. Treatment with 6-mercaptopurine was discontinued after 26 days because of nausea and vomiting. U-6538 administration was concluded 2 days later because the patient could no longer maintain an adequate oral intake of fluids. He had received 50 to 100 mg. of chlorpromazine daily during the previous 4 months. The following day he became jaundiced, febrile, had a severe hemorrhage from the tumor, and expired. He had shown no antitumor effect nor signs of riboflavine deficiency on the combination of U-6538 and 6-mercaptopurine. He received 49.8 gm. of U-6538 in 60 days. Laboratory examinations were essentially unchanged during the study except for a rise in serum alkaline phosphatase from 2.8 to 9.8 Bessey-Lowry units and fluctuation of the hemoglobin concentration because of hemorrhages. At *post-mortem* examination he had a necrotic tumor in the right nasopharynx extending posteriorly to the cervical vertebrae, focal hepatitis, pericholangitis, focal areas of tubular necrosis in the kidneys, and a bacteremia due to *P. aeruginosa*.

Patient #3.—A 54-year-old white female had a mole excised from her back which had been diagnosed as melanoma 3 years before admission. The first metastases were noted about 1 year later. On admission she had numerous cutaneous and subcutaneous metastases, generalized lymphadenopathy, and hepatomegaly. Roentgenograms of the chest and bones showed multiple pulmonary and osseous lesions.

Laboratory studies were essentially normal except for a cephalin-flocculation test (Hanger) of two plus. She received triethylenethiophosphoramide parenterally at intervals of 1 week for a 3-month period. There was transient hematopoietic depression but no evident influence on tumor growth. When the trial of U-6538 was begun, the patient had a bromsulphalein retention of 11 percent in 45 minutes and a serum alkaline phosphatase of 4.3 Bessey-Lowry units. She was given 200 mg. of U-6538 intramuscularly every 8 hours for 34 days. An occasional dose was omitted when the patient's fluid intake was poor. She gradually developed edema of the lower extremities so the administration of the drug was discontinued. She received 25.2 gm. of U-6538 in 58 days. No antitumor effect nor evidence of riboflavine deficiency was noted. During the study the serum alkaline phosphatase rose to 8.0 Bessey-Lowry units, the serum albumin fell to 1.4 gm. per 100 ml. and proteinuria developed. The patient had progressive hepatic and pulmonary insufficiency and expired 2 weeks after the last dose of U-6538 had been received. *Post-mortem* examination showed extensive melanoma metastases, particularly in the bones, lungs, and liver. There was a brown pigment in the Kupffer cells of the liver and in the renal tubules with adjacent necrosis of some of the tubular epithelium.

Patient #4.—A 43-year-old white female developed subcutaneous masses near a mole on her leg 1 year before admission. These masses and the mole were excised and the diagnosis of melanoma was made. A tumor soon reappeared near the excision scar and in the inguinal area on the same side. On admission there were nodular subcutaneous masses in the left leg and groin. Three weeks later pulmonary metastases and cutaneous metastases on the trunk had appeared. She received Mechlorethamine parenterally at intervals of 1 week for a 3-month period, with no evidence of effect on the tumor. The patient became dyspneic and developed vertigo, a homonymous hemianopsia, and hepatomegaly. At this time the hemoglobin was 8.5 gm. per 100 ml., the leukocyte count was 3,200 cells per mm.^{3} and the serum albumin was 2.2 gm. per 100 ml. She was placed on a riboflavine-deficient diet and had the usual preliminary toxicity studies with U-6538. She then received 100 mg. of U–6538 intramuscularly every 8 hours for 15 days. She developed crystalluria several times and some doses were omitted. Then she received 50 mg. every 8 hours for 13 days. Again some doses were omitted when crystalluria occurred. The patient received 6.7 gm. of U-6538 over a period of 47 days. Because of abdominal distention, nausea, and vomiting, she was maintained on parenteral fluids with no vitamins or protein for 1 week. At that time, bleeding of the lips, cheilosis, erythema of the tongue, seborrhea behind the ears, and a diffuse erythematous skin eruption developed. Thiamin, pyridoxine, and nicotinic acid, but not riboflavine, were administered. The skin eruption subsided, but the patient became comatose and expired after several grand mal seizures 3 days later. The serum albumin had fallen to 1.6 gm. per 100 ml. and the platelet count to 98,000 per mm.^{3} *Post-mortem* examination showed extensive melanoma metastases to the brain, liver, lungs, and mesentery of the bowel.

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Effect of Concurrent Feeding of Tween 80 on the Carcinogenicity of Orally Administered 3-Methylcholanthrene[1,2,3]

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Summary

The results of this study indicate that Tween 80, when fed concurrently with methylcholanthrene, potentiates the local as well as distant carcinogenic activity of methylcholanthrene. This potentiation was evidenced by 1) an increase in the incidence and severity of tumors of the forestomach and proliferative changes of the glandular stomach, 2) an increase in the total tumor incidence in tissues distant from the gastrointestinal tract, and 3) an increase in the incidence of multiple primary neoplasms in individual animals.—**J. Nat. Cancer Inst. 22: 363–399, 1959.**

Tweens are a series of nonionic surface-active agents containing sorbitan and polyoxyethylene groups combined with various fatty acids. As such, they are both hydrophilic and lipophilic and have properties that are characteristic of "wetting agents" and emulsifiers. In recent years these substances have been the subject of considerable study because of their potential use in foods and medicinal preparations. Previous papers from this laboratory (*1*, *2*) summarized these studies and indicated that under certain conditions dietary Tween 20 may increase iron absorption from the intestinal tract. More recently, other studies have indicated the possible cocarcinogenic properties of Tweens. Ekwall *et al.* (*3*), in 1951, reported the ability of Tweens to enhance the penetration of a carcinogen, 3,4-benzpyrene,[5] into the stomach wall of mice and cats. In 1956, Setälä (*4*) reported the cocarcinogenic effect of Tweens in the induction of skin tumors in mice. He demonstrated, in a skin-painting experiment, that the carcinogen 9,10-dimethyl-1,2-benzanthracene,[6] when dissolved in Tweens, is capable of producing a higher incidence of skin tumors than when it is dissolved in an inert vehicle such as paraffin oil.

[1] Received for publication August 19, 1958.

[2] Supported in part by Public Health Service grant C-2657.

[3] Former designation: 20-methylcholanthrene.

[4] The authors wish to express their appreciation to Mrs. Loraine Petrie and Mr. Toby Guinsatao for their technical assistance, and to Mrs. Ivy Annamunthodo for her help in preparing the manuscript.

[5] *Chemical Abstracts'* nomenclature: benzo[e]pyrene.

[6] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[a]anthracene.

Our present investigation is concerned with the study of two possible effects of Tween-feeding in relation to carcinogenesis: First, its effect on the activity of an orally administered carcinogen on the gastrointestinal tract mucosa, that is, its local cocarcinogenic effect, if any; and second, its effect on the absorption and transportation of an orally administered carcinogen across the gastrointestinal mucosa, as judged by the incidence of neoplasms in tissues distant from the gastrointestinal tract.

Both aspects of this investigation have important relationships to previous observations by others. For example, Lorenz and Stewart (*5–8*) have shown, in their extensive studies, that feeding other "wetting agents" enhances the carcinogenic activity of dietary methylcholanthrene and dibenzanthracene on the forestomach of mice. That Tweens may influence the gastrointestinal absorption of phenanthrene-like substances is indicated by the work of Kellner *et al.* (*9*) as well as that of Payne and Duff (*10*). Both of these studies demonstrated that dietary Tween 80 enhanced the gastrointestinal absorption of cholesterol by rabbits. Oil that was fed with the cholesterol appeared to augment this effect.

In the present study, 3-methylcholanthrene, a phenanthrene-related carcinogen, was fed to mice, with and without Tween 80, in high fat diets. The chronic effect of Tween 80 on the carcinogenicity of 3-methylcholanthrene was evaluated by the difference in tumor incidences among the various diet groups.

Materials and Methods

In this experiment, male C57BL mice about 19 to 20 weeks old and weighing between 20 to 30 gm. were used. The mice were divided into 4 groups, each of which was fed a specific diet, as shown in table 1. The detailed compositions of the individual diets are given in table 2.

In preparing the diets containing methylcholanthrene, special care was taken to insure an even distribution of the small amount of carcinogen in the diets. This was accomplished by adding the specified amount of methylcholanthrene to the liquid ingredients of the diet and then heating the mixture to approximately 100° C. until all the carcinogen was dissolved. The oily solution thus obtained was then added to the dry ingredients of the diet and blended with a mechanical mixer. Rubber gloves were worn by all personnel involved in handling the carcinogenic diets.

The amount of diet given to each mouse daily was 2 gm., a quantity representing the maximum amount of daily diet which could be consumed constantly and equally by all groups of mice over a prolonged period, as determined in a preliminary experiment. Throughout the study, this amount of diet was fed daily and uneaten diet was discarded. Water was given *ad libitum*.

With a daily dietary intake of 2 gm., the amount of methylcholanthrene consumed by each mouse in groups II and IV was 0.6 mg., while the amount of Tween 80 consumed by each mouse in groups III and IV was 100 mg.

All 4 groups of mice were fed their specified experimental diets for the first 10 weeks of the experiment. They were then fed the basal diet for

TABLE 1.—*Diets for various groups*

Group	Number of mice	Experimental diet
I	23	Basal diet
II	27	Basal diet + methylcholanthrene
III	28	Basal diet + Tween 80
IV	27	Basal diet + methylcholanthrene + Tween 80

the subsequent 8 weeks (weeks 10 to 18). Following this, all 4 groups were taken off the basal diet and fed Purina laboratory chow *ad libitum* for the remainder of the experiment. Animals that died before the termination of the experiment were autopsied. Heart, lungs, stomach, small intestine, large intestine, liver, spleen, and kidneys were taken routinely for histologic examination. The stomach was routinely opened along the greater curvature and spread out in its entirety on a piece of cardboard for fixing. Later on, multiple longitudinal sections were taken for histologic examination. Other tissues, such as the skin, were studied microscopically only when gross pathologic changes were observed at the time of autopsy. All tissues were fixed in neutral buffered formalin, embedded in paraffin, and stained routinely with hematoxylin and eosin. Certain special stains, including Congo red for amyloid and the periodic acid-Schiff stain for glycogen, were used in some instances. In the 51st week of the experiment, all surviving animals were killed with ether and autopsied, and the same tissues as mentioned previously were taken for histologic examination.

TABLE 2.—*Detailed composition of experimental diets for various groups*

Dietary components	Grams per 100 grams of diet			
	Group I	Group II	Group III	Group IV
Casein	10	10	10	10
Dextrin	40	40	40	40
Sucrose	10	10	10	10
Salt mixture (O&M)	4	4	4	4
Dried Brewer's yeast	5	5	5	5
L-Cystine	0.5	0.5	0.5	0.5
Alphacel	10	10	5	5
Cottonseed oil	19.5	19.5	19.5	19.5
Wheat-germ oil	1	1	1	1
Tween 80	—	—	5	5
Oleum percomorphum	1 drop	1 drop	1 drop	1 drop
3-Methylcholanthrene	—	0.03	—	0.03
Total	100.0	100.03	100.0	100.03

Results

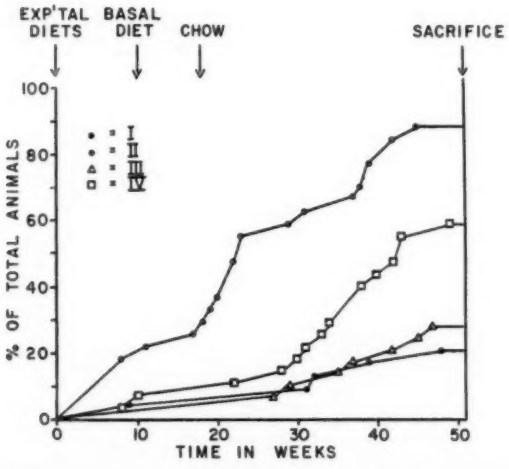
Preliminary Observations

A preliminary experiment revealed that continuous feeding of the carcinogen-containing diets to mice for more than 4 months resulted in

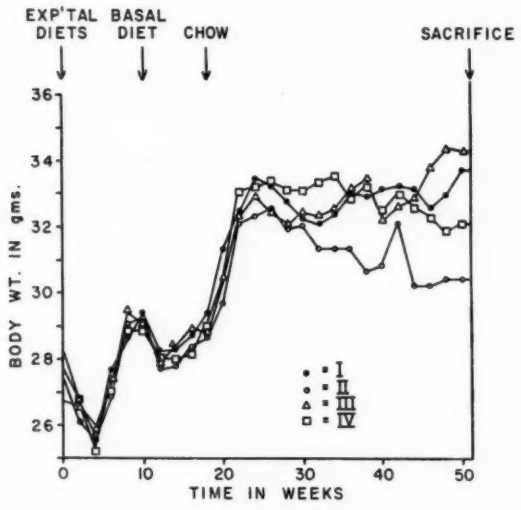
a high degree of early mortality in these animals. The few survivors eventually developed anasarca and died in approximately 6 months, or shortly thereafter. Ascites was particularly prominent. In some of these cases, the ascitic fluid was clear. In others, the ascitic fluid was thick, white, opaque, and had a chylous appearance. Ascitic fluid of intermediate composition also occurred. In a few cases of chylous ascites, there was associated bilateral chylous effusion into the pleural cavities. Similar findings have been reported by Lorenz and Stewart (*5*) in an experiment involving oral administration of dibenzanthracene and methylcholanthrene to mice in the form of olive-oil emulsions. This complication led to the experimental plan adopted in the present investigation, which consisted of feeding the carcinogenic diets for approximately 2 months only. The following observations were made in the definitive experiment:

Mortality, Body Weight, and Diet Consumption

As judged from the curves in text-figures 1 and 2, dietary methylcholanthrene produced an increased mortality, but the addition of Tween 80 to the diets at a level of 5 percent seemed to have no deleterious effect on the mortality or the body weight of the animals. The unusually high mortality of group II in the early part of the experiment, especially after the 8th week, was associated primarily with the development of severe amyloidosis in such vital organs as the liver, spleen, and kidneys. A *Salmonella* infection, with multiple hepatic abscesses, was also observed in some of these animals dying in the early part of the experiment. But, in general, the incidence of concurrent infection and amyloidosis was not great enough to indicate any relationship between the two in the present experiment.



TEXT-FIGURE 1.—Mortality rate of various groups.



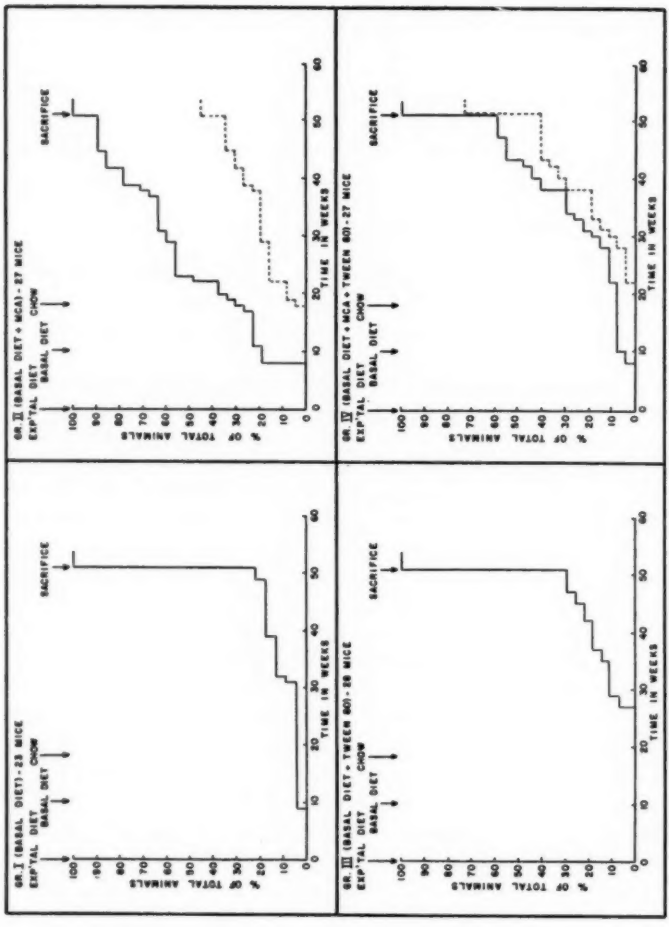
TEXT-FIGURE 2.—Average body weight plotted against time.

Data on dietary consumption were not obtained for the present experiment, but similar data collected from preliminary experiments showed that there was no significant difference in the dietary consumption between group II and group IV animals, indicating that the amount of carcinogen consumed by these 2 groups was approximately the same. The difference in the incidences of neoplastic changes between these 2 groups was therefore in all probability due to factors other than the amount of carcinogen consumed.

Since, until the termination of the experiment, tissues were taken for histologic studies only after the animals had died, the so-called time of appearance of any pathologic lesion obviously depended to a considerable extent on the time of death of the animal bearing the particular lesion. Similarly, the apparent incidence of a pathologic lesion at any specified interval of time depended heavily on the incidence of mortality at the time. In order to avoid these difficulties in interpretation, it would have been necessary to conduct an experiment in which animals were killed periodically for histologic examination. Since this experiment was not conducted on such a basis, interpretation of the frequency of various pathologic findings was made with reference to the mortality incidence of the animals at the time.

Lung Tumors

Animals in groups I and III, which were not fed methylcholanthrene in their diets, were found to be completely free of lung neoplasms; whereas animals in groups II and IV, which had methylcholanthrene or methylcholanthrene plus Tween 80 in their diets, developed multiple tumors in their lungs (text-fig. 3). These lung tumors were histologically identical


GR. I (BASAL DIET) - 23 MICE
EXP'TAL DIET
BASAL DIET
CHOW
SACRIFICE
GR. II (BASAL DIET + MCA) - 27 MICE
GR. III (BASAL DIET + TWEEN 80) - 28 MICE
GR. IV (BASAL DIET + MCA + TWEEN 80) - 27 MICE
% OF TOTAL ANIMALS
TIME IN WEEKS
CUMULATIVE MORTALITY
CUMULATIVE LUNG TUMOR INCIDENCE

TEXT-FIGURE 3.—Mortality and lung-tumor incidence plotted as percent of total animals against time.

to those observed by Grady and Stewart (*11*), as well as Lorenz and Stewart (*5*), following subcutaneous injection or feeding of dibenzanthracene or methylcholanthrene to mice.

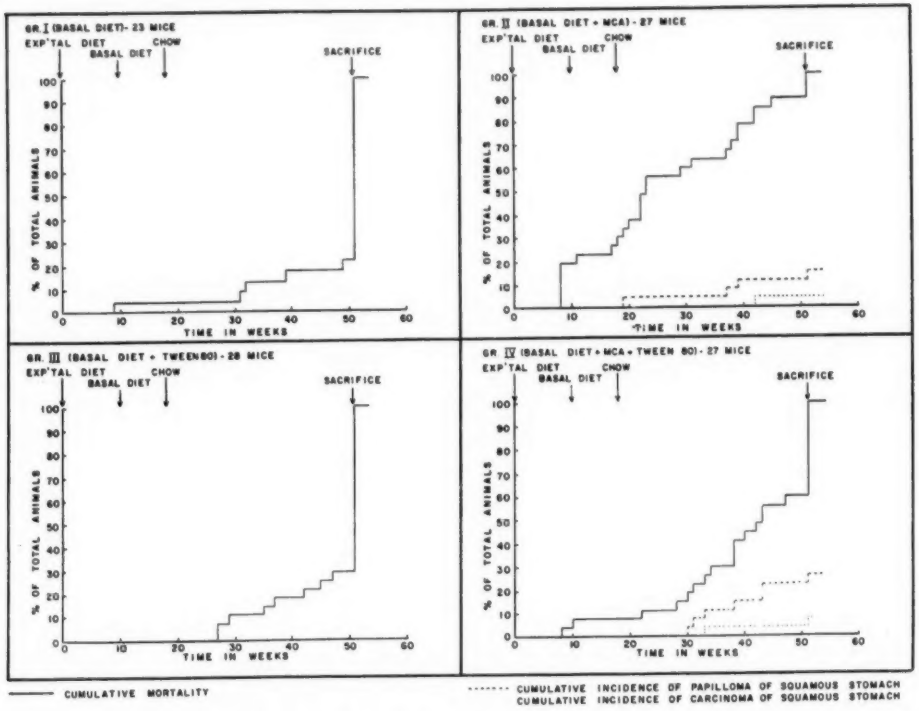
Microscopic examination of the smaller lung tumors showed a solid mass of cuboidal tumor cells derived apparently from the alveolar lining (fig. 1). Some of the larger tumors were better differentiated and showed a papillary structure (fig. 2). The individual papillae were composed of thin connective-tissue cores covered by cuboidal or low-columnar tumor cells. The size of the tumor increased with time so that some of the tumors occasionally occupied an entire lobe of a lung. Frequently, the larger tumors also showed an increase in mitotic figures, suggesting accelerated growth. The total number of such tumors per pair of lungs likewise increased with time, so that toward the end of the experiment it was not unusual to find as many as 50 to 100 such tumors in the lungs of a single animal.

By comparing groups II and IV (text-fig. 3), we see that, starting with 27 mice in each group, the total incidence of lung tumors at the end of the experiment was 44.4 percent in group II and 74.1 percent in group IV, both expressed as percent of total animals. To be sure, more animals in group II died during the initial period of the experiment when there was insufficient time for the lung tumors to develop, thus leaving fewer animals to develop these tumors later on in the experiment (text-fig. 3). Nevertheless, it is obvious, by comparing the curves of group II and group IV, that in the last half of the experiment, when for a period before the animals were killed the incidence of mortality in the 2 groups was very similar, the incidence of lung tumors in group IV remained greater than that in group II.

The first lung tumor of group II was found in the 18th week, while that of group IV was found in the 22d week (text-fig. 3). However, this difference in the time of appearance of the lung tumors may be more apparent than real, because a closer examination of the curves in text-figure 3 will show that the mortality incidences at about this period of time were markedly different in the 2 groups. Whereas the mortality incidence of group II from week 18 to week 22 was 18.5 percent, the mortality incidence of group IV in the same interval of time was 3.7 percent. If more animals in group IV had succumbed during this period of time, we might have discovered the first lung tumor in group IV at an earlier date.

Tumors of Squamous Stomach

Mice in groups I and III, which were not fed methylcholanthrene, were found to be free of papillomas and carcinomas of the squamous stomach (text-fig. 4). On the other hand, mice in groups II and IV, which had methylcholanthrene or methylcholanthrene plus Tween 80 in their diets, did develop these lesions (text-fig. 4, figs. 3 and 4). Tween 80 added to methylcholanthrene did not seem to reduce the induction time for squamous papilloma of the forestomach, as the first such lesion was found in


GR. I (BASAL DIET)- 23 MICE
EXP'TAL DIET
BASAL DIET
CHOW
SACRIFICE
% OF TOTAL ANIMALS
TIME IN WEEKS
GR. II (BASAL DIET + MCA) - 27 MICE
GR. III (BASAL DIET + TWEEN 80) - 28 MICE
GR. IV (BASAL DIET + MCA + TWEEN 80) - 27 MICE
CUMULATIVE MORTALITY
CUMULATIVE INCIDENCE OF PAPILLOMA OF SQUAMOUS STOMACH
CUMULATIVE INCIDENCE OF CARCINOMA OF SQUAMOUS STOMACH

TEXT-FIGURE 4.—Mortality and incidence of papilloma and carcinoma of squamous stomach plotted as percent of total animals against time.

the 19th week in group II and in the 30th week in group IV (text-fig. 4), although here, too, as with the lung tumors, the marked difference in mortality incidences of the 2 groups from about the 19th to the 30th week might have misled us. The final incidence of squamous papilloma was 14.8 percent in group II and 25.9 percent in group IV. From text-figure 4, we see that the incidence of squamous papilloma followed incidence of the mortality much more closely in group IV than in group II.

The final incidence of carcinoma of the squamous stomach was 3.7 percent in group II and 7.4 percent in group IV (text-fig. 4). Since these percentages represented 1 animal and 2 animals, respectively, they were not thought to be statistically significant.

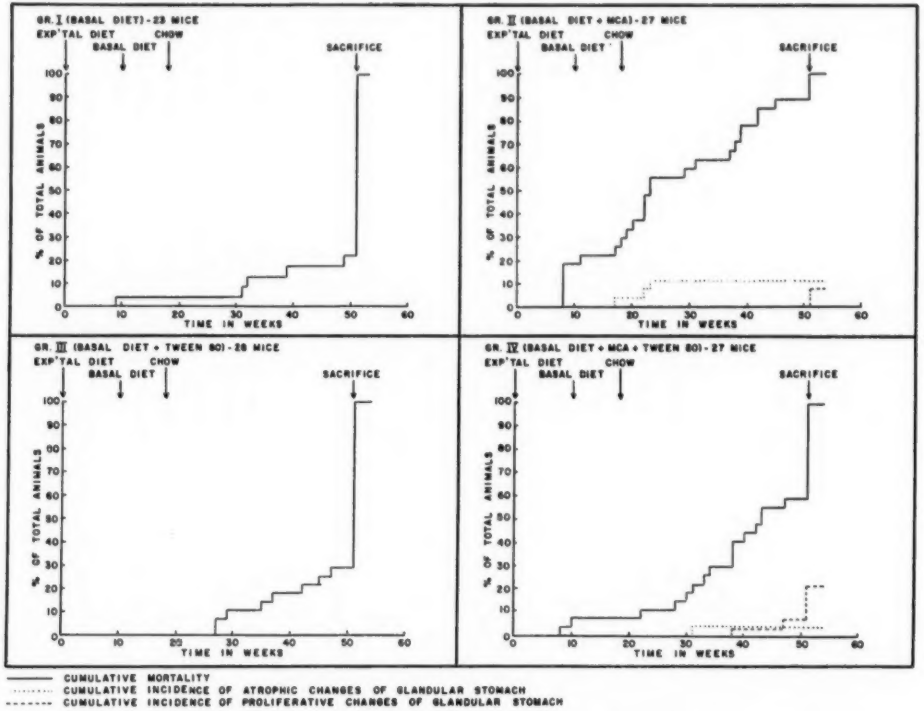
Mucosal Changes in Glandular Stomach

Mice in groups I and III, which were not given methylcholanthrene, had no pathologic changes in the glandular stomach throughout the experiment (text-fig. 5). A typical histologic picture of the glandular stomach from mice of groups I and III is shown in figure 5. Mice from groups II and IV, the diets of which contained methylcholanthrene or methylcholanthrene plus Tween 80, showed both atrophic and proliferative changes in the glandular stomach (text-fig. 5).

The atrophic changes consisted of a decrease in the thickness of the mucosa, loss of parietal cells, replacement of normal gastric glands by mucus-secreting glands of the intestinal type, and a slight to moderate increase of fibrous tissue in the mucosa, muscularis mucosae, and submucosa. An example of the atrophic changes is shown in figure 6. These changes, whenever present, were uniformly found throughout the entire length of the multiple longitudinal sections routinely taken of each stomach, and were never observed in similar sections of the stomach from control animals.

The proliferative changes consisted of an increase in the thickness of the mucosa, at times to as much as 4 times the normal thickness (figs. 7 and 8). The glands here were also of the intestinal type, but instead of being short and atrophic, hypertrophy and hyperplasia had taken place, giving rise to tortuosity of the glands and disruption of the normal regularity of the glandular pattern. The glands were frequently hyperchromatic and showed crowding and depolarization of the nuclei. There was often an apparent increase in the number of mitotic figures. As with atrophic changes, a loss of parietal cells was observed. Cystic degeneration of the glands was also seen (fig. 8).

As shown in text-figure 5, the appearance of atrophic changes preceded the appearance of proliferative changes in both group II and group IV animals. In fact, the appearance of the atrophic changes seemed to be limited to the earlier part of the experiment, while the appearance of the proliferative changes seemed to be limited to the later part. The course of action of the carcinogen, judging from the sequence of pathologic changes observed here, would seem to be an initial suppressive effect followed by a late enhancing effect on cell growth. The result was that


GR. I (BASAL DIET) - 23 MICE
EXP'TAL DIET
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% OF TOTAL ANIMALS
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GR. II (BASAL DIET + MCA) - 27 MICE
GR. III (BASAL DIET + TWEEN 80) - 26 MICE
GR. IV (BASAL DIET + MCA + TWEEN 80) - 27 MICE
CUMULATIVE MORTALITY
CUMULATIVE INCIDENCE OF ATROPHIC CHANGES OF GLANDULAR STOMACH
CUMULATIVE INCIDENCE OF PROLIFERATIVE CHANGES OF GLANDULAR STOMACH

TEXT-FIGURE 5.—Mortality and incidence of atrophic changes and proliferative changes of glandular stomach plotted as percent of total animals against time.

the mucosa of the glandular stomach went through an initial phase of atrophic changes, together with a conversion of the highly specialized gastric glands to the simple intestinal type of glands, and then progressed to a final phase of proliferative changes. A similar sequence of events has been observed in the induction of hepatomas by acetylaminofluorene, in which suppression of the liver cells was first observed, followed by proliferation. The same sequence of changes has been observed frequently in cases of irradiation dermatitis which eventually progress to carcinomas of the skin.

As seen in text-figure 5, Tween 80 added to methylcholanthrene did not cause the atrophic changes of the glandular stomach to appear earlier in group IV than in group II, nor did it cause an increase in the incidence of atrophic changes in group IV. In fact, the incidence of atrophic changes in group II (11.1%) was higher than that in group IV (3.7%). However, as pointed out earlier, the mortality incidence in group II was much greater than that in group IV in the initial part of the experiment. Since the appearance of atrophic changes was limited to the earlier part of the experiment, the apparently lower incidence of atrophic changes in group IV may well have been because fewer animals in this group died early in the experiment. Thus, even though there may have been atrophic changes, they would not have been detected but would have progressed to the proliferative changes that were discovered in the later part of the experiment.

The proliferative gastric changes in group IV (text-fig. 5) did occur earlier and in greater number than in group II (week 38 *vs.* week 51). This earlier appearance of proliferative changes in group IV may not have been significant, since the number of animals involved was small. Furthermore, the greater incidence of proliferative changes in group IV may have been partially explained by the greater number of animals surviving in the last half of the experiment. However, it probably could not be explained entirely on this basis, for there was a relatively long period before the end of the experiment during which the animals in the 2 groups were dying at about the same rate, yet proliferative changes of the glandular stomach were found only in the animals of group IV. The final incidence of these proliferative changes was 22.2 percent in group IV and 7.4 percent in group II. In addition to this difference in the incidences, it should be added that the final proliferative changes observed in the animals that were killed were generally more severe histologically in group IV than in group II.

One interesting finding in connection with the proliferative changes of the glandular stomach was observed in an animal in group IV that was killed. In the glandular stomach of this animal there was, in addition to severe proliferative changes in the mucosa, an invasion of the glands below the muscularis mucosae (fig. 9). Around the invaded areas, the submocosa was infiltrated with chronic inflammatory cells consisting of lymphocytes and plasma cells. This type of "adenomatous lesion" has been observed to develop spontaneously in mice of strain I (*12*). It has

also been seen in mice fed methylcholanthrene and dibenzanthracene in oil emulsions (*8*).

Leukemias and Other Neoplasms

The leukemias, both lymphocytic and myelogenous, and other tumors found in this experiment are listed in table 3. Leukemia occurred most frequently in group IV, with 4 animals developing this disease, or 14.8 percent of the group. Of these 4 animals, 3 had myelogenous leukemia and 1 had lymphocytic leukemia. This neoplastic process occurred next most frequently in group II, with 2 animals developing the myelogenous variety and 1 developing the lymphocytic variety. Together they represented 11.1 percent of the animals in the group. In group III, there was 1 animal developing myelogenous leukemia and 1 developing a malignant thymoma. The latter demonstrated characteristics of a lymphocytic leukemia, as evidenced by a large quantity of abnormal lymphocytes in the blood. Group I had the lowest incidence of leukemia, with 1 animal developing the lymphocytic variety. In view of the small difference in the incidences of leukemia in the various groups and its seemingly spontaneous appearance in animals of the 2 control groups, little significance was attached to these findings.

As shown in table 3, hepatomas were found only in group III (Tween diet) and group IV (Tween + methylcholanthrene), with 1 animal in each group developing this tumor. Both of these hepatomas could very well have been on the basis of spontaneous occurrence. It should be pointed out, however, that they differed considerably from one another in histologic characteristics. The hepatoma of group III had comparatively well-differentiated cells with nuclei that were very uniform in size. There was only slight nuclear hyperchromatism. No other changes in the nuclei were observed. The hepatoma of group IV, on the other hand, showed a great deal of variation in nuclear and cell size. There were abnormal mitoses with varying degrees of apparent polyploidy of the chromosomes. Hyperchromatic giant nuclei were present. Nuclear "inclusion bodies," probably of viral origin, were found in great numbers in the tumor cells. These "inclusion bodies" were also observed in the liver of animals without hepatoma, particularly those animals receiving methylcholanthrene. They will be described.

One hemangioendothelioma was found in a group IV animal in the region of the pancreas. It was not found in animals of any other group.

Squamous-cell carcinoma of the skin developed in 3 animals of group II (11.1%) and in 2 animals of group IV (7.4%). In addition, there was a benign sebaceous-gland tumor of the skin developing in an animal of group II. It seems likely that these tumors developed as a result of contact of the skin with the carcinogenic diet rather than as a result of absorption of carcinogen from the gastrointestinal tract, as most of these tumors developed at sites of denuded skin caused by fighting among the animals. The males of C57BL strain are pugnacious animals that fight and bite each other. Many open skin wounds resulted from such be-

TABLE 3.—*Incidence and time of appearance of leukemias and other neoplasms*

			Group I—23 mice	Group II—27 mice	Group III—28 mice	Group IV—27 mice
Leukemia	Incidence	Number of mice	1	3	1	4
		Percent of total mice	*4. 3*	*11. 1*	*3. 6*	*14. 8*
	Time found		Weeks: 49	Weeks: 22, 23, 51	Weeks: 29	Weeks: 28, 31, 33, 47
Malignant thymoma becoming leukemia	Incidence	Number of mice	0	0	1	0
		Percent of total mice	0	0	*3. 6*	0
	Time found				Weeks: 45	
Hepatoma	Incidence	Number of mice	0	0	1	1
		Percent of total mice	0	0	*3. 6*	*3. 7*
	Time found				Weeks: 51	Weeks: 51
Hemangioendothelioma	Incidence	Number of mice	0	0	0	1
		Percent of total mice	0	0	0	*3. 7*
	Time found					Weeks: 51
Squamous-cell carinoma of skin	Incidence	Number of mice	0	3	0	2
		Percent of total mice	0	*11. 1*	0	*7. 4*
	Time found			Weeks: 22, 23, 31		Weeks: 28, 38
Benign, sebaceous-gland tumor of skin	Incidence	Number of mice	0	1	0	0
		Percent of total mice	0	*3. 7*	0	0
	Time found			Weeks: 51		

havior in the early part of the experiment. A few of these wounds were unusually large and did not heal properly. As time went on, a majority of the squamous-cell carcinomas developed at such sites because the animals had a tendency to stay in the food cup while they were eating, thereby allowing contact of the denuded skin with the carcinogenic food particles.

Incidence of Multiple Primary Neoplasms in Individual Animals

As summarized in table 4, none of the animals in groups I or III developed more than 1 primary neoplasm. On the other hand, a number of animals in groups II and IV developed more than 1 primary neoplasm at a time. In group II there were 6 animals (22.2%) with multiple primary neoplasms. Each of these animals had 2 primaries. In group IV there were 10 animals (37.0%) with multiple primary neoplasms. Of these, 8 had 2 primaries and 2 had 3 primaries. It should be added that some of these multiple primary neoplasms were also associated with atrophic or proliferative changes of the glandular stomach. The skin tumors were not included in the compilation of the table.

TABLE 4.—*Incidence of multiple primary neoplasms in individual mice**

Group	Two primary neoplasms		Three primary neoplasms	
	Number of mice	Percent of total mice	Number of mice	Percent of total mice
I	0	0.0	0	0.0
II	6	22.2	0	0.0
III	0	0.0	0	0.0
IV	8	29.6	2	7.4

*Skin tumors excluded.

Tumor Incidence in Various Groups

The tumor incidence per animal, excluding skin tumors, is summarized in table 5 for the various groups. Group IV had the highest tumor incidence per animal (1.30); next, group II (0.74), group III (0.11), and group I (0.04), respectively. It is notable that the tumor incidence per animal in group IV is 1.76 times that in group II.

Liver Nuclear Changes

It was observed, in examining the histologic sections of liver, that there were changes in the hepatic nuclei of animals in groups II and IV which were not found in animals of groups I and III (figs. 10 to 12b). These changes did not begin to occur until 5 to 6 months after the start of the experiment. Thereafter, they increased in severity with time. These changes consisted of:

Abnormal mitoses.—Abnormal mitoses, resulting in irregular distribution of chromosomes and probable polyploidy, were seen in some of the liver sections from animals of groups II and IV.

TABLE 5.—*Total tumor incidence per group and per animal**

Group	Mortality and neoplasia	Incidence of mortality and various neoplasms at different time intervals expressed as number of animals						Total tumor incidence	Total No. of animals	Tumor incidence per animal
		Time in weeks								
		0 to 10	10 to 20	20 to 30	30 to 40	40 to 50	Sacrifice: 50 to 51			
I	Mortality	1	0	0	3	1	18		23	
	Leukemia	0	0	0	0	0	1	1		0.04
II	Mortality	5	5	6	5	3	3		27	
	Lung tumor	0	2	3	2	2	3	12		
	Papilloma of squamous stomach	0	1	0	2	0	1	4 (20)		0.74
	Carcinoma of squamous stomach	0	0	0	0	1	0	1		
	Leukemia	0	0	2	0	0	1	3		
III	Mortality	0	0	3	2	3	20		28	
	Leukemia	0	0	1	0	0	0	1		
	Malignant thymoma	0	0	0	0	1	0	1 (3)		0.11
	Hepatoma	0	0	0	0	0	1	1		
IV	Mortality	2	0	3	7	4	11		27	
	Lung tumor	0	0	3	5	3	9	20		
	Papilloma of squamous stomach	0	0	1	3	2	1	7		
	Carcinoma of squamous stomach	0	0	0	1	0	1	2 (35)		1.30
	Leukemia	0	0	1	2	1	0	4		
	Hepatoma	0	0	0	0	0	1	1		
	Hemangioendothelioma	0	0	0	0	0	1	1		

*Skin tumors excluded.

Variation in size of cells and nuclei.—Variation in the size of the hepatic cells and nuclei in group II and group IV animals had a general tendency to increase proportionately with the length of time that the animals had lived, with some of the cells and nuclei progressing to giant size and others diminishing to a smaller size than at the beginning of the experiment (fig. 11). This wide range in size was most probably dependent on aberrations in mitoses, which led to unequal partition of chromosomes in the daughter cells.

Variation in shape of nuclei.—The variation in the shape of the nuclei observed in this experiment seemed to result mainly from amitotic division of the cells, which occurred with unusual frequency in the livers of group II and group IV animals, leading to dumbbell-shaped nuclei or nuclei with polycyclic borders (fig. 12).

These nuclear changes had no statistical correlation with infectious processes or amyloidosis occurring in the liver.

In addition to these changes, there were intranuclear inclusion bodies in the liver cells of all 4 groups of animals, though a definitely greater number was present in the liver of group II and group IV animals. These intranuclear inclusion bodies were found about equally often in group II and group IV animals and were believed to be of viral origin (figs. 13 and 14). The number of these intranuclear inclusion bodies, according to the above observations, would seem to be in some way related to the administration of methylcholanthrene. This brings to mind the theory concerning the etiology of cancer that all exogenous carcinogens, including carcinogenic chemicals, work through viruses in the induction of tumors, as suggested by Rous (*13*) and Andrewes (*14*) and supported recently by Oberling and Guérin (*15*). However, the fact that hepatomas were not found any more frequently in the 2 groups of animals fed methylcholanthrene, although these animals had a greater number of viral inclusion bodies in their liver, and the fact that no inclusion bodies were found in the hepatoma of the group III animal (Tween diet), although they were found in great numbers in the hepatoma of the group IV animal (methylcholanthrene + Tween), seem to indicate that these viruses were not connected etiologically with the development of hepatomas in the present experiment. On the other hand, it seems more reasonable to conclude that these exogenous viruses were probably much better able to infect the hepatic cells after the latter were in some way altered by the action of methylcholanthrene. This would explain the presence of a greater number of these inclusion bodies in the hepatic cells of animals fed methylcholanthrene, whether these cells were in a hepatoma or in an ordinary liver.

Amyloidosis

An important incidental finding in this experiment was the frequent development of widespread amyloidosis. The organs most commonly

involved were spleen, liver, kidneys, small intestine, stomach, and cecum, in that order.

The spleen, liver, and kidneys were usually involved concurrently in the process, though occasionally amyloid was found only in the spleen or the liver. In the spleen (fig. 15), the amyloid was characteristically deposited initially around the periphery of the lymph follicles, later on spreading to involve diffusely the entire spleen. In the liver (fig. 16), the amyloid was deposited between the endothelial lining cells of the sinusoids and the hepatic cells, as well as in the wall of the blood vessels. In the kidneys, the amyloid was deposited in the glomeruli and blood vessel walls. In the glomeruli, the amyloid was found between the basement membrane and the endothelial lining of the capillaries (fig. 17).

Amyloidosis of the gastrointestinal tract, as observed in this experiment, could exist alone or concurrently with severe widespread amyloidosis of the other organs. In the small intestine, the amyloid deposit was almost uniformly found in the lower jejunum or ileum (fig. 18) and never in the duodenum. The stomach was occasionally the site of involvement. The cecum, on the other hand, was only rarely involved. In all these sites, the amyloid was deposited in the lamina propria of the mucosa. The muscle coat never contained amyloid. The small intestine was usually more severely involved than the other gastrointestinal sites. The villi here were usually expanded as a result of the deposit of amyloid in the lamina propria (fig. 18). Amyloidosis was also occasionally observed in the pancreas; here the deposit was mainly in the connective tissue between the acini. As the pancreas was not among the tissues taken routinely for histologic examination, the relative frequency of amyloidosis in this organ could not be assessed. In one animal amyloidosis was found in the heart alone.

Amyloidosis was found with about equal frequencies in all groups of animals in this experiment, with the exception of group II, which had about 65 percent of its animals involved with this process. In other groups, approximately 50 percent of the animals were involved. The high incidence of amyloidosis with severe renal involvement seen in group II may have been responsible for the unusually high mortality of this group in the early part of the experiment. No explanation was evident for the high incidence of amyloidosis in this group. Amyloidosis was observed beginning at about the 8th week of the experiment. Thereafter it was found at all time intervals of the experiment. Its severity did not seem to have any particular correlation with the length of time that the animals had lived.

Three possible factors may have contributed to the amyloidosis observed in this experiment:

> *Infection.*—Amyloidosis of the secondary variety is known to be associated with long-term, chronic infectious processes. In this experiment, there seemed to be no connection between the development of amyloidosis and the sporadic cases of *Salmonella*

infection with chronic hepatic abscesses found in some of the animals.

Tumor-bearing state.—Amyloidosis of the secondary variety has been observed in terminal cancer patients as well as in tumor-bearing mice. In this experiment, the lack of correlation between the over-all tumor incidence and the incidence of amyloidosis in the various groups does not support the tumor-bearing state as the primary causative factor for amyloidosis.

Casein in diets.—Amyloidosis has been produced by subcutaneous injection of sodium caseinate (*16*) as well as by feeding casein to mice (*17*). The latter means of producing amyloidosis was reported to be less predictable than the former (*17*). All the diets in the present experiment contained 10 percent casein. The fact that 3 out of the 4 groups of animals had about the same incidence of amyloidosis seemed to favor dietary casein as the primary causative factor of amyloidosis in this experiment.

Extramedullary Hematopoiesis

Extramedullary hematopoiesis, predominantly the myeloid variety, was observed in the liver and spleen of about 25 percent of the animals in groups II and IV. The cause for this condition was not clear, though it could conceivably arise as a result of methylcholanthrene toxicity on the bone marrow. This speculation could not be proved or disproved in the present experiment, since the bone marrow was not studied histologically. However, Lorenz and Stewart (*5*), who fed methylcholanthrene to mice in the form of olive-oil emulsions continuously for about a year, found no appreciable histologic changes in the bone marrow. On the other hand, these investigators (*5*) did find a variable degree of marrow depletion, involving all cell types, in mice fed dibenzanthracene in the same kind of emulsions for 5 months or more.

Discussion

From these experimental results, the following generalizations can be made:

Tween 80, when fed concurrently with methylcholanthrene, potentiates the latter's carcinogenic activity, in comparison to methylcholanthrene alone, as evidenced by 1) a higher total tumor incidence, 2) a higher incidence of multiple primary neoplasms, and 3) more pronounced proliferative changes of the glandular stomach.

Among the individual tumors that make up the total tumor incidence in the present experiment are: lung tumor, squamous papilloma and squamous carcinoma of the forestomach, hepatoma, leukemia, malignant thymoma, and hemangioendothelioma. Of these, the difference between the lung-tumor incidences of animals fed the 2 different diets provides the strongest and most consistent evidence for the ability of Tween 80 to enhance the carcinogenic activity of methylcholanthrene. Differences

in other tumor incidences, though perhaps not statistically significant when taken individually, do provide a significant trend when taken as a whole and point to the ability of Tween 80 to increase the incidence of tumors produced by methylcholanthrene. In the absence of any evidence that Tween 80 is itself a carcinogen, one must conclude from the results of the present investigation that Tween 80 acts, in part at least, as a cocarcinogen.

Thus far, in interpreting the data, it has been assumed that all the tumors induced, with the exception of skin tumors, were due to carcinogen absorbed from the gastrointestinal tract. The occurrence of skin tumors raises the question as to whether the skin contributed to the absorption of carcinogen by virtue of its contact with carcinogenic food particles and consequently played a part in the induction of internal tumors. This question is particularly valid in view of the fact that lung tumors such as those observed in this experiment have been produced by some investigators by painting carcinogen on the skin (*18*). The fact that only a small number of skin tumors was produced in comparison to the number of lung tumors and the fact that the majority of the lung tumors induced were unaccompanied by any malignant or premalignant changes of the skin by no means rule out the possibility of the skin as an important organ for the absorption of carcinogen under the conditions of the present experiment. Considerable experimental evidence definitely indicates that carcinogenic hydrocarbons absorbed from their initial site of application may not induce any tumors in the immediate vicinity of the site of application, but may instead evoke tumors in susceptible tissues in a wholly different part of the body. A notable example of this is the work of Magnus (*19*), who attempted to produce gastric cancer in mice by introducing dibenzanthracene in olive-oil emulsions directly into the stomach. No gastric tumors developed, but tumors of the lungs appeared in 95 percent of the animals, as against 4 to 8 percent of the controls. In the results obtained in our study, there was absolutely no correlation between the lung-tumor incidence and the skin-tumor incidence within the individual groups of animals consuming the carcinogenic diets. Furthermore, the skin tumors were most frequently observed at the denuded areas of skin caused by the fighting of the animals. These facts would indicate that the skin tumors and the internal tumors were not induced by carcinogen derived from the same route, and that the absorption of carcinogen through the skin was not a significant factor in the induction of internal neoplasms in this experiment.

Assuming that all tumors, except those of the skin, were induced as a result of the absorption of carcinogen from the gastrointestinal tract, there are at least 3 mechanisms by which Tween 80 may act to increase the incidence of tumors produced by methylcholanthrene:

1) By virtue of its surface-active properties, Tween 80 is capable of bringing about a better contact of methylcholanthrene with the gastrointestinal tract mucosa. The enhancing effect of surface-active agents on the gastrointestinal action of carcino-

genic hydrocarbons was pointed out early by Lorenz and Stewart (*5–8*). They demonstrated, for example, that ingestion of olive-oil emulsions of methylcholanthrene and dibenzanthracene by mice resulted in a high incidence of carcinoma of the small intestine. In contrast, carcinoma of the forestomach developed only rarely. However, when the olive-oil emulsions were made acid-stable by the addition of a surface-active agent, the dioctyl ester of sodium sulfosuccinic acid (Aerosol OT), squamous carcinoma of the forestomach developed with increasing frequency. The explanation for this phenomenon offered by the investigators was that the "wetting agent" permitted intimate and prolonged coating of the squamous epithelial lining by the carcinogen.

Whereas squamous-cell carcinoma of the forestomach can be produced with the aid of surface-active agents, adenocarcinoma of the glandular stomach has not been so produced. The difficulty of bringing the carcinogenic agent into intimate contact with the mucus-covered glandular stomach was suggested in 1939 by Van Prohaska, Brunschwig, and Wilson (*20*) as the primary cause for their failure to induce gastric adenocarcinoma by feeding methylcholanthrene to mice. Since then, other investigators (*21–25*) have made similar postulations from work with such other experimental animals as dogs. This concept of the existence of a "protective mucous barrier" gained experimental support when it was found that implantation or injection of carcinogenic agents directly into the wall of the glandular stomach, thus bypassing the so-called mucous barrier, resulted in the production of adenocarcinoma (*26*). In our experiment, an attempt was made to study the ability of Tween 80 to bring about surface contact of methylcholanthrene with the glandular stomach despite this mucous barrier. No adenocarcinoma with proved metastases was produced by this means. However, as stated earlier, there were proliferative changes in the gastric glandular mucosa as well as invasion of the glands below the muscularis mucosae. The significance of these lesions could not be fully assessed in the present experiment, as the regional lymph nodes were not taken for histologic examination. Furthermore, there was no way of predicting how far these lesions would have progressed had the animals survived longer.

2) By virtue of its emulsifying action and its ability to bring about surface contact, Tween 80 is able to increase the absorption and transportation of methylcholanthrene across the gastrointestinal tract mucosa, such that this carcinogenic agent is available in greater quantity for the induction of tumors at sites distant from the gastrointestinal tract.

3) Tween 80, if absorbed with methylcholanthrene, could conceivably facilitate the penetration of methylcholanthrene into cells of target tissues distant from the gastrointestinal tract,

such as the lungs. Krantz *et al.* (*27*) indicated that approximately 5 percent of the Tween 80 ingested orally by humans is in some way absorbed and metabolized by the body. Since only a very small concentration of Tween is needed to solubilize carcinogenic hydrocarbon, as shown by Ekwall *et al.* (*3*), it is not inconceivable that the small amount of Tween 80 absorbed would, by virtue of its surface-active properties, facilitate the penetration of methylcholanthrene into the cells of target tissues distant from the gastrointestinal tract, thus leading to an increased incidence of tumors in these tissues.

The effect of Tween 80 on the "induction time" for various tumors caused by methylcholanthrene is not consistent enough for one to draw any conclusion. As has been pointed out earlier, the so-called induction time for a tumor in this investigation depended on when the animal bearing the particular lesion died. A better way to evaluate the effect of Tween 80 on tumor induction time would be to kill the animals periodically during the course of the experiment.

Thus far, few studies have been carried out on the absorption, metabolism, and excretion of orally administered methylcholanthrene. However, studies on related hydrocarbons show that, in general, when this kind of compound is ingested, most of it is passed unchanged in the feces, part of it is absorbed, and the absorbed hydrocarbon is partly excreted in the bile and urine, and partly retained in certain tissues, such as adipose tissue and intestinal wall. From our results a number of speculations can be made concerning the route of absorption of methylcholanthrene.

When methylcholanthrene is absorbed from the intestinal tract, there are 2 possible routes by which it can reach the general circulation: 1) It can be carried in the blood stream through the portal system into the systemic venous circulation, or 2) it can be carried in the lymph through the thoracic duct into the systemic venous circulation. If the first route is the main one, there might be a high incidence of hepatomas, for the liver would be the first target organ of the methylcholanthrene absorbed from the intestinal tract. On the other hand, if most of the methylcholanthrene enters the thoracic duct, one would expect a high incidence of lung tumors, for the lungs would then be the first target organs for the absorbed methylcholanthrene, excluding the heart. The unusually high incidence of lung tumors in this experiment (74.1% of the animals in group IV and 44.4% in group II), together with the remarkably low incidence of hepatomas (0% of the animals of group II and 3.7% in group IV), certainly suggests that methylcholanthrene is absorbed primarily through the lymphatics. However, the high incidence of lung tumors and the low incidence of hepatomas could simply be a reflection of the marked difference in susceptibility of the 2 organs to methylcholanthrene. On the other hand, if one assumes that the methylcholanthrene in the intestine is absorbed through the lymphatics, one could explain the occurrence of chylous ascites and chylothorax in mice fed methylcholanthrene over a prolonged period. This phenomenon was

observed in one of our preliminary experiments as well as by Lorenz and Stewart (*5*). A possible explanation is as follows:

It is known that the methylcholanthrene molecule has an affinity for, both fat and protein. In the lymphatics that drain the intestinal tract, the fat content of the lymph is high. The methylcholanthrene absorbed therefore tends to remain dissolved in fat. As the lymph from the intestine drains into the thoracic duct, however, its fat content is gradually diluted by the lymph drained from the thoracic region. As a result, the methylcholanthrene may come out of solution and become attached to the protein of the endothelial-lining cells of the lymphatics. Since methylcholanthrene is a cytotoxic agent, it may damage the endothelial cells and cause fibrosis in the wall of the lymphatics. If methylcholanthrene is fed continuously over a long period, the fibrosis thus produced could be severe enough to cause an obstruction of the thoracic duct. As a result, there may be leakage of lymph from the lymphatics proximal to the site of obstruction due to back pressure. It should be recalled that chylous ascites and chylothorax were observed only in animals fed methylcholanthrene over a period of 6 months or more. They were not observed in animals fed methylcholanthrene for 3 months or less. The absence of chylous ascites and chylothorax in the latter animals could have been due to the fact that, as the carcinogen in the diet was withdrawn early, the fibrosis it caused in the thoracic duct would not be severe enough to cause obstruction. It is notable that similar findings of chylous ascites and chylothorax were reported by Lorenz and Stewart (*5*) in mice in which oral administration of dibenzanthracene and methylcholanthrene in the form of olive-oil emulsions was prolonged. No explanations were given by these investigators as to why the chylous ascites or chylothorax occurred, although spectrographic analysis of the ascitic fluid from animals fed dibenzanthracene revealed no trace of this compound in the unmetabolized form.

In view of the fact that cholesterol, a structurally similar compound, has been demonstrated to be absorbed from the intestine via the lymphatics (*28*, *29*), it seems likely that methylcholanthrene would be absorbed in the same fashion.

In conclusion, it should be mentioned that gastric cancer is essentially a human disease, occurring rarely in animals (*30–32*). However, recently, a high incidence of spontaneous carcinoma of the glandular stomach has been reported in a South African rodent (*33*). Although carcinomas have been induced experimentally in the squamous cell-lined forestomach of mice by feeding carcinogenic compounds, adenocarcinomas of the glandular stomach have not been so produced. Thus far, only "hyperplastic" or "adenomatous" lesions have been induced in the stomachs of mice (*8*) and monkeys (*34*) by the feeding method. Nevertheless, the theory that gastric cancer is caused by alimentary carcinogens, perhaps acting on extraordinarily highly susceptible cells, is still the most attractive. It is for this reason that all the experimental efforts thus far expended are justified, no matter how unsuccessful they seem at the present.

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PLATES

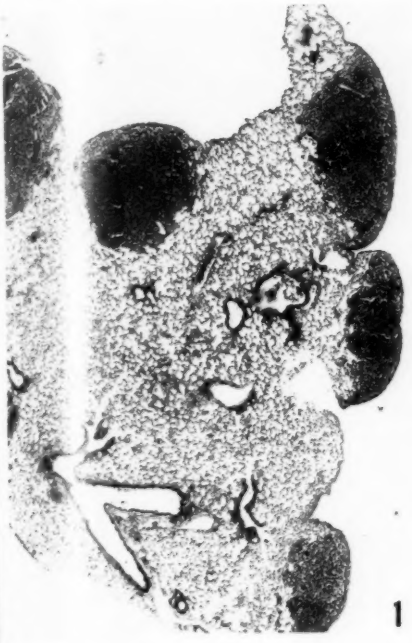
PLATE 28

FIGURE 1.—Multiple lung tumors, in early stage of development, from group IV animal dying in 38th week of experiment. These tumors are derived from alveolar lining cells rather than from bronchial epithelium. *Note* multicentric nature of the tumors and their predilection for growing in peripheral zones of lung. The individual tumors are composed of solid masses of cuboidal tumor cells. × 22

FIGURE 2.—Lung tumor, in late stage of development, from group IV animal killed at end (51st week) of experiment. *Note* papillary structure of a portion of the tumor. × 78

FIGURE 3.—Squamous papilloma of forestomach from group II animal killed at end of experiment. × 34

FIGURE 4.—Squamous carcinoma of forestomach from group IV animal killed at end of experiment. *Note* invasion of the squamous epithelium to a considerable distance below the muscularis mucosae. The overlying squamous epithelium is highly proliferative. × 37



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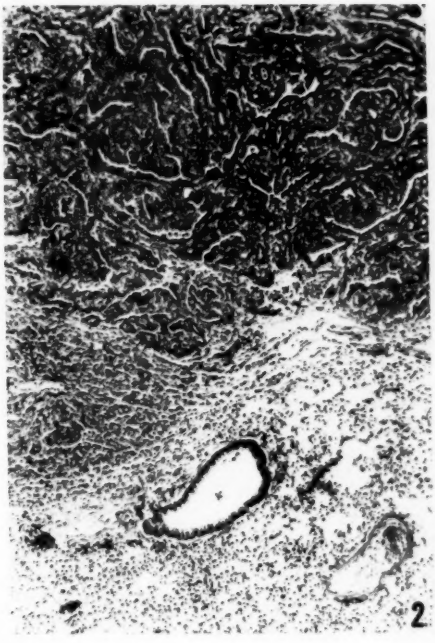

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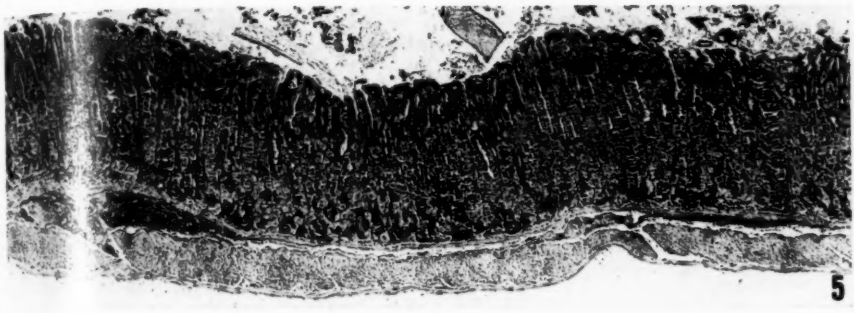
PLATE 29

FIGURE 5.—Glandular stomach from group I animal killed at end (51st week) of experiment. The parietal cells are present throughout entire length of the mucosa. × 65

FIGURE 6.—Atrophic changes of glandular stomach from group IV animal dying in 31st week of experiment. *Note* decrease in thickness of the mucosa (*cf.* fig. 5, which is a section of the glandular stomach from a group I animal under same magnification). *Note also* loss of parietal cells and replacement of the normal gastric glands by mucus-secreting glands of the intestinal type. × 65

FIGURE 7.—Proliferative changes of glandular stomach from group IV animal killed at end of experiment. The thickness of the mucosa is about 3 times that of control (*cf.* fig. 5). *Note* highly proliferative intestinal-type of glands, especially in basal layer of the mucosa; distortion of normal glandular pattern; hyperchromatism; and loss of parietal cells. × 65



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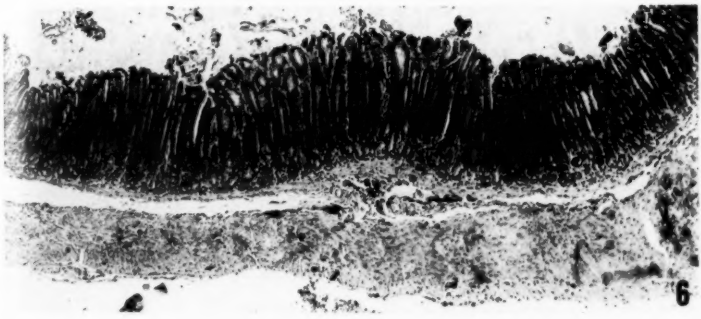

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PLATE 30

FIGURE 8.—Proliferative changes of glandular stomach from group IV animal dying in 38th week of experiment. The thickness of the mucosa is 3 to 4 times that of control (*cf.* fig. 5, which is a section of glandular stomach from a group I animal under the same magnification). *Note* hyperchromatic proliferative glands, with cystic changes and loss of parietal cells. × 65

FIGURE 9.—Proliferative changes of glandular stomach from group IV animal killed at end (51st week) of experiment. *Note* the almost polypoid proliferation of mucosa. *Note also* that, in addition to having those changes listed under figures 7 and 8, there are invasions of the glands below the muscularis mucosae at three sites. At one of these sites, the invading glands are more proliferative and irregular than those at the other sites. × 54

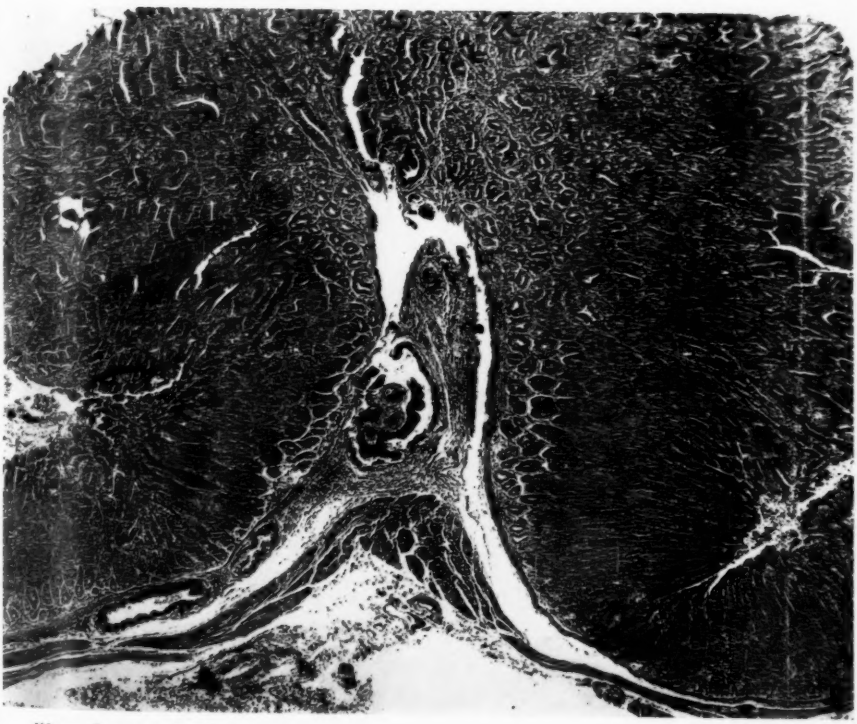

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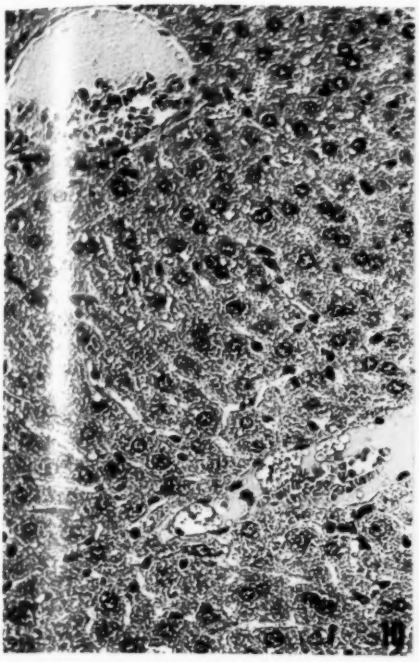
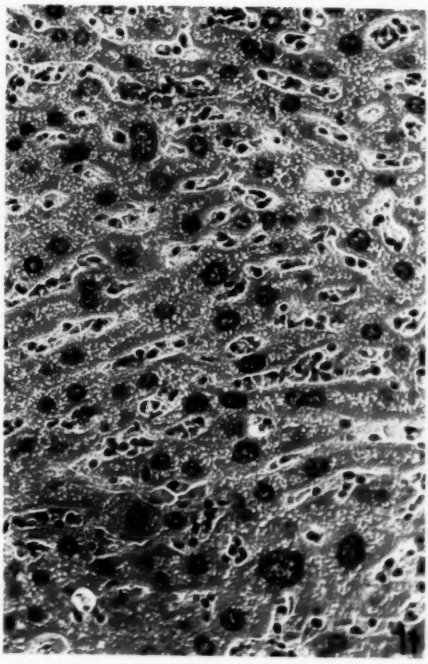
PLATE 31

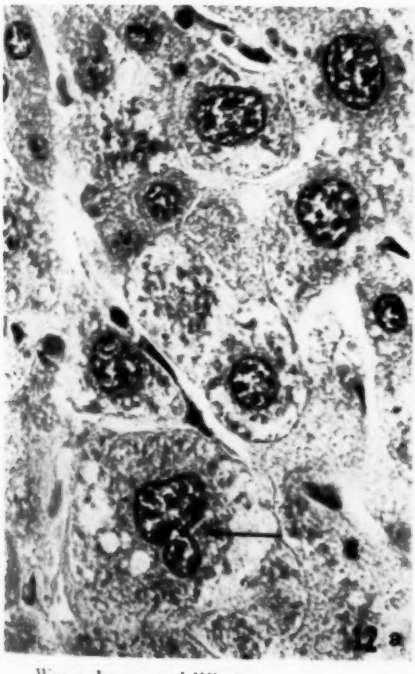
FIGURE 10.—Liver from group I animal killed at end (51st week) of experiment. *Note* regularity in size of cells and nuclei. × 290

FIGURE 11.—Liver from group IV animal dying in 27th week of experiment. *Note* marked variation in size of hepatic nuclei. *Compare* this with liver from group I animal in figure 10 with respect to nuclear size, taken under same magnification. × 290

FIGURES 12A AND 12B.—Figure 12a is from liver of group IV animal killed at end of experiment, and figure 12b is from liver of group II animal dying in 36th week of experiment. *Note* variation in shape of nuclei, especially at sites of *arrows*.







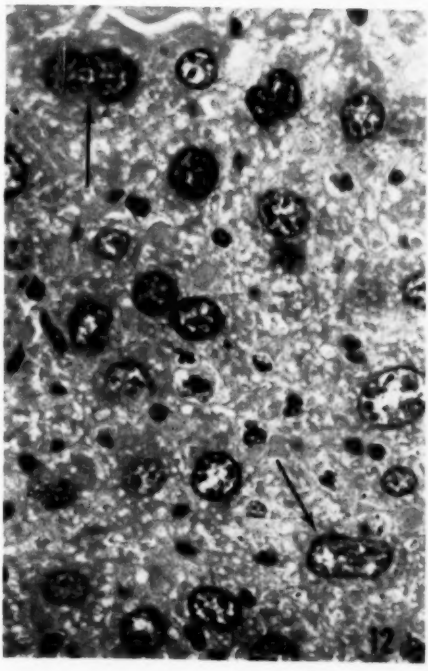
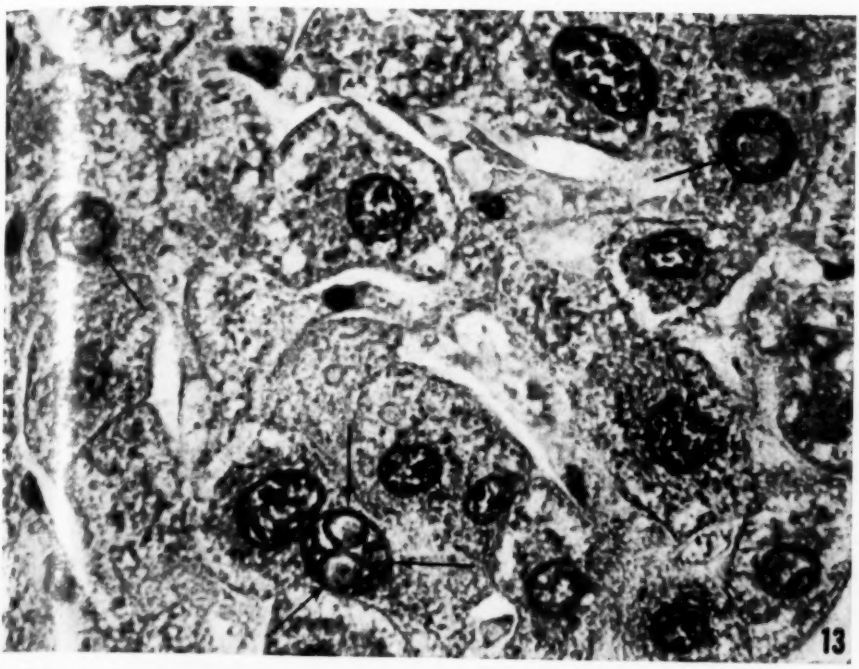


PLATE 32

FIGURE 13.—Liver from group IV animal killed at end (51st week) of experiment. *Note* intranuclear inclusion bodies at sites of *arrows*. × 950

FIGURE 14.—Periodic acid-Schiff stain of a hepatoma section from group IV animal killed at end of experiment. *Note* that some intranuclear inclusion bodies are positive for the stain (marked *a*); others are partially positive (marked *b*); still others are almost completely negative (marked *c*). × 885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A
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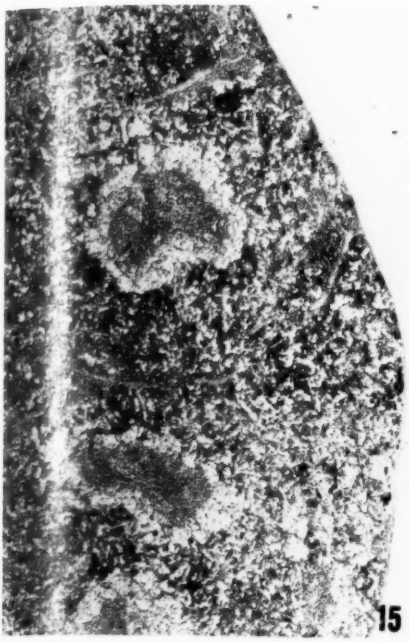
PLATE 33

FIGURE 15.—Amyloidosis of spleen from group II animal dying in 8th week of experiment. *Note* perifollicular distribution of the amyloid deposit. There is also amyloid in the red pulp. × 37

FIGURE 16.—Amyloidosis of liver from a group II animal dying in 8th week of experiment. *Note* deposition of amyloid 1) between endothelial lining cells of sinusoids and hepatic cells, and 2) in wall of blood vessels. × 81

FIGURE 17.—Amyloidosis of kidney from group IV animal killed at end (51st week) of experiment. *Note* deposition of amyloid inside glomerulus, between basement membrane and endothelial lining of the capillaries. In this instance, the amyloid deposits are somewhat nodular in appearance. × 495

FIGURE 18.—Amyloidosis of small intestine (ileum) from group III animal dying in 29th week of experiment. *Note* deposition of amyloid in lamina propria, causing expansion of tips of villi, giving them a clublike appearance. × 78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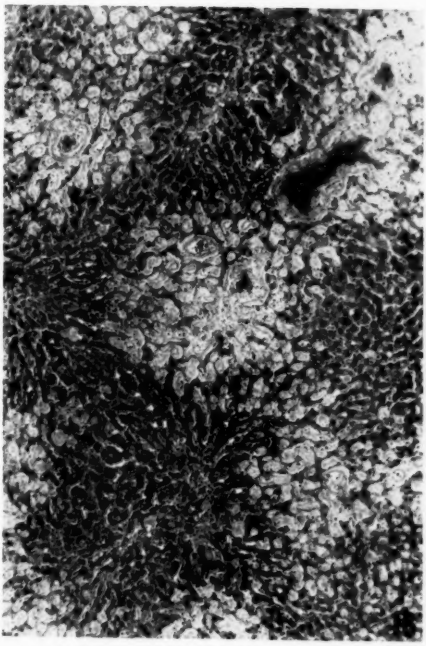

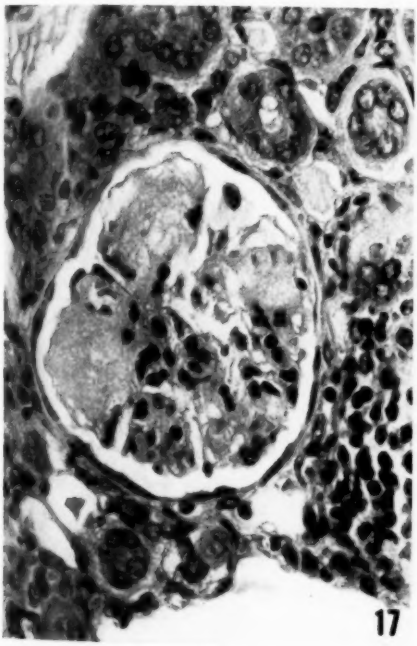
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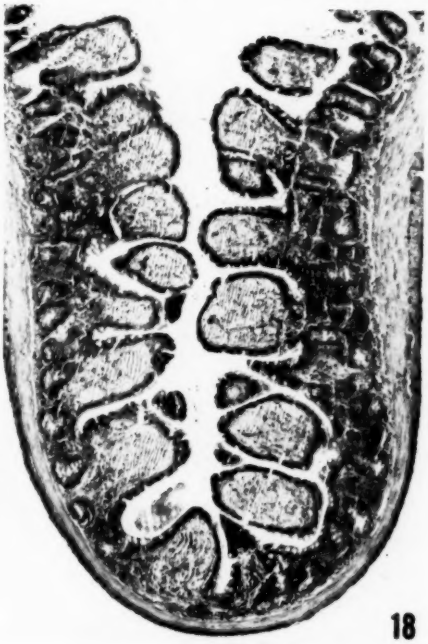
18

Carcinogenic Activity of Cigarette-Smoke Condensate. I. Effect of Trauma and Remote X Irradiation [1,2]

FRED G. BOCK *and* GEORGE E. MOORE,[3] *Roswell Park Memorial Institute, Buffalo, New York*

Summary

In Swiss mice painted with cigarette-smoke condensate, benign and malignant skin tumors were produced in all groups, regardless of coincident irritation. The data indicate that qualitative differences in laboratory results probably are not due to minor differences in skin-painting techniques but may be due to differences in mouse stocks employed. In Swiss female and C3H male mice, intense irradiation of a small area increased the sensitivity of distant areas of skin to painting with cigarette-smoke condensate. In this case, X irradiation apparently acted as a systemic initiating agent of carcinogenesis. The Swiss female mice were much more sensitive to the combined treatment than were the C3H males.—J. Nat. Cancer Inst. 22: 401–411, 1959.

In 1953 Wynder *et al.* (*1*) reported they produced tumors by applying cigarette-smoke condensate to mouse skin. Although Wynder and his colleagues duplicated their work qualitatively and extended it to other species (*2*), other workers failed to confirm their observations (*3–5*).

Many studies relating irritation to carcinogenic processes have been published since Deelman (*6*) observed that wound healing accentuated tumor production. Inconsistencies in the experimental results (*7*, *8*) led Berenblum (*9*) to propose that the variation was due to timing of the irritation. He suggested that irritation could exert a promoting effect after carcinogenic change was initiated by other agents. No evidence exists to indicate that the differences observed in tobacco-tar studies are due to the minor irritation caused by painting or shaving. However, we were persuaded that controlled investigation of this possibility should be undertaken, inasmuch as Wynder's experiments have stimulated use of mouse skin to measure carcinogenicity of tobacco products. The experiments described here were conducted to determine whether the divergent results could have been due to differences in painting techniques, and also

[1] Received for publication August 25, 1958.

[2] Supported in part by a grant from the Tobacco Industry Research Committee.

[3] The authors are indebted to Mr. Melvin Reinhard and Mr. James Carlson for suggestions and guidance concerning irradiation factors. Mrs. Dolores Mickens was of great assistance in much of the technical work.

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to determine whether use of secondary agents would make screening tests for carcinogens more sensitive.

Since the beginning of these experiments, successful induction of mouse-skin tumors by painting with cigarette-smoke condensate has been reported by Englebreth-Holm and Ahlmann (*10*), by Orris *et al.* (*11*), and by Passey *et al.* (*12*). The study reported here serves to confirm a growing body of evidence that cigarette smoke is indeed a mouse-skin carcinogen and to emphasize the importance of using a variety of techniques when a "weak" carcinogen is studied.

Materials and Methods

Almost all the "tar" used in this experiment was produced in a manifold-type smoking machine with a falling water column as the negative pressure source. A 35-cc., 2-second puff was taken once each minute until the butts were approximately 23 mm. long. This machine differed significantly from that used by Wynder in regard to frequency, volume, and other characteristics of the puff. The pressure *versus* time curve for the puff resembled a positively skewed curve instead of the plateau-type curve used by Wynder and by Orris *et al.* A small amount of tar was produced by a manifold-type machine with a plateau-type pressure *versus* time curve.

Cigarette smoke, condensed at −20° C. was washed from the collection flasks weekly, with the use of the smallest convenient volume of acetone. The acetone solution was condensed under reduced pressure. The condensed tar was redissolved in 15 ml. of acetone per original carton of cigarettes to give solution "A." Since the recovery of "tar" usually was about 4 gm. per carton, solution "A" was approximately 20 percent "tar."

Experiment A was designed to determine the effect of variations in painting technique and the effect of irritation upon the experimental results. It was also intended to determine whether a variation in dose rate would produce a large difference in the nature of the response. For this purpose, Swiss female mice were divided into 8 groups as follows:

Group I: 30 mice shaved on alternate Mondays just before painting with "A."

Group II: 30 controls treated as above, but painted with acetone.

Group III: 30 mice shaved the first Friday of each month, but not painted until the following Monday; painted with "A."

Group IV: 30 mice shaved when it appeared necessary; painted with "A."

Group V: 30 mice rubbed with sandpaper 3 times weekly just before painting with "A." The "rubbing" was just vigorous enough to induce a slight degree of bleeding (fig. 1).

Group VI: 10 controls treated like group V, but painted with acetone.

Group VII: 30 mice that were never shaved; painted with "A."

Group VIII: 10 control mice that were never shaved; painted with acetone.

The entire backs of the mice were shaved with an Oster electric clipper (size 0000 head). The back of each animal was "painted" with 0.25 ml. of solution "A" using a 0.25 ml. syringe. The viscosity and volume of the solution were such that it flowed evenly over the entire shaved area. The animals of groups I, II, III, V, VI, VII, and VIII were painted 10 times a week, while animals of group IV were painted 5 times a week.

To produce solution "B," the condensed tar was treated with 1 volume of acetone and 8 volumes of petroleum ether or heptane. The upper nonpolar layer was decanted away from the aqueous residues and washed with 1 N hydrochloric acid until the washings had a pH less than 3. The heptane was removed under reduced pressure, and the residues were dissolved in acetone to make a final volume of 3 ml. per original carton of cigarettes. This procedure was intended to concentrate a fraction that would contain the bulk of the polynuclear aromatic hydrocarbons.

Experiment B was conducted with 5 groups of mice as follows:

Group IX: 50 nonirradiated Swiss female mice; painted with "B."
Group X: 65 irradiated Swiss female mice; painted with "B."
Group XI: 39 irradiated Swiss female mice serving as controls for group X; painted with acetone.
Group XII: 45 irradiated C3H males; painted with "B."
Group XIII: 19 irradiated C3H males, controls for group XII; painted with acetone.

Physical radiation factors were HVL 0.1 mm. Al (100 kv. unfiltered) and a dose rate of 780 r per minute at a distance of 20 cm. A single dose of 2500 r was delivered when the animals were 55 days of age. At this time the dorsal hair was in a resting stage of growth. The animals were placed in individual lead containers with wall thicknesses of 1/16- to 1/8-inch, in each of which there was a 1 cm.^{2} window permitting radiation of 1 cm.^{2} of the dorsum of the mouse just anterior to the tail (fig. 2). With this dose of 2500 r delivered to the skin, the dose scattered to the interior of the mouse under the lead shield was found to be 2.5 r.

Experimental animals were "painted" 5 times a week with 0.25 ml. of solution "B" by the same method used for solution "A." Controls were painted with a similar volume of acetone. To keep the hair short, all the animals were shaved as needed.

All animals in experiments A and B were painted continuously until they died or were moribund. Without knowing the experimental groups from which the skin lesions arose, we examined the lesions microscopically. The first skin tumors appeared in about 9 months. Fifty percent of the mice lived 15 months or longer after the start of the experiment.

Results and Discussion

The data are presented in tables 1 and 2. Tumors were considered carcinomas when the cells invaded the panniculus carnosus. Metastases to the lung and reginal lymph nodes were frequent, while metastases to the liver and other organs were rare (figs. 4 through 7).

TABLE 1.—*Effect of painting technique and irritation on response to cigarette-smoke condensate*

Group	Number of mice living 6 months	Number of mice with skin cancer	Number of mice with carcinomas or papillomas	Mice with skin tumors (*percent*)
I—shaved biweekly immediately before painting	28	1	6	21
III—shaved monthly 3 days before painting	28	1	5	18
IV—shaved "when necessary"	28	2	8	29
V—"sandpapered" immediately before painting	27	7	10	37
VII—never shaved	30	5	9	30
Total of experimental groups	*141*	*16*	*38*	*27*
II—controls for group I	30	0	0	—
VI—controls for group V	10	0	0	—
VIII—controls for group VII	10	0	0	—

TABLE 2.—*Effect of mouse stock and remote X irradiation on response to cigarette-smoke condensate*

Group	Number of mice living 6 months	Mice with skin tumors after 64 weeks		Number of tumors÷number of mice in expt. (×100)	Number of skin cancers observed histologically
		Number	Percent		
IX—Swiss painted only	49	13	27	31	2
X—Swiss irradiated and painted	65	44	68	125	17
XI—Swiss irradiated controls	36	0	—	—	—
XII—C3H irradiated and painted	42	12	29	33	1
XIII—C3H irradiated controls	19	0	—	—	—

Dose Level of Cigarette Tar Used in Experiment A

Mice of group IV, which were painted only 5 times a week, gave a response similar to that of the animals painted 10 times a week. Likewise, in animals that were never shaved the tumor yield was comparable to that in shaved animals, even though the full-length hair reduced the exposure of the underlying skin. These data suggest that the dose rate employed in experiment A was maximal. Such results would be expected if the inactive residue of tar applied to the animal was of sufficient

volume to interfere with the absorption of additional quantities of active material. On the other hand, the data from experiment B disclose that purification of crude cigarette tar permitted increased exposure of the target cells to carcinogen, probably because a smaller volume of inactive residue was applied with a greater amount of active material. In this case there was less interference with movement of the agent to the sensitive cells. In this regard it is of interest to note the experiments of Twort and Twort (*13*) who found that dibenz[*a*,*h*]anthracene was less active on mouse skin when applied in mineral oil than when applied in chloroform, a volatile solvent which would leave no residue on the skin after its application.

Effect of Irritation on Results

No qualitative differences in the biologic response to cigarette smoke was demonstrated by any of the groups of experiment A. The total tumor yield was comparable in all groups. While the number of carcinomas varied from one group to another, the number is too small to warrant drawing conclusions.

Two experiments comparable to that of group V have been conducted by other workers. Ludford (*14*) scarified mice with sandpaper before and after painting them with coal tar. He found that scarification of the painted area decreased the number of observed tumors, while painting after scarification produced a slight retardation in the development of cancer. On the other hand, in an experiment by Riley and Pettigrew (*15*), mice brushed with stiff bristles, after being painted with dibenz[*a*,*h*]-anthracene, had more tumors than did the controls which were painted only. The design of each of these experiments differs from that of group V in the timing of the irritation and in the intensity of the agent. Accordingly, a comparison of our data with that already published is probably unwarranted. Still, it appears that the quantitative differences observed by investigators studying cigarette-smoke condensate are not due to small differences in the method of tar application.

Effect of Irradiation

The results of experiment B are presented in table 2. Nearly all tumors observed in the irradiated animals appeared in areas that *were shielded* from the direct X-ray beam. The irradiated area usually was covered with a scar which was quite apparent, even in albino mice. A comparison of the mouse of figure 2 with that of figure 3 demonstrates this effect.

The effect of local irradiation upon the development of tumors in distant areas of skin cannot be explained on the basis of present data. Mottram (*16*) found that X irradiation increased the number of papillomas produced in skin previously painted with benzo[*a*]pyrene. In this respect irradiation acted as a "promoting agent." Likewise Shubik *et al.* (*17*) observed that exposure of mouse skin to the β-ray emission of Th^{204} produced tumors when the skin was painted with croton oil after

irradiation. In this instance, irradiation possessed "initiating action." In both investigations, however, the effect of X rays could be ascribed to direct action upon the affected cells.

Taschner, Gottlieb, and Spritzer (*18*) irradiated mice with 650 r of X rays while protecting a small area of skin. Subsequently, painting with methylcholanthrene produced more tumors in the protected area of irradiated animals than in nonirradiated controls. Unfortunately, details of the quality of irradiation and exact location of tumors in relation to the irradiated area are not available in their report. However, the data of Taschner *et al.* appear to be qualitatively similar to those obtained by us.

In our experiment, cells which eventually became malignant were beneath the lead shield and received a very low dose of radiation—of the order of 2.5 r. Tobacco tars, when applied after benzo[*a*]pyrene, have been reported by Hamer and Woodhouse (*4*) and by Gellhorn (*19*) to possess mild cocarcinogenic activity. However, since tobacco tar alone can give rise to skin tumors, neither these results nor our own eliminate the possibility of an additive carcinogenic effect. Accordingly, two hypotheses can be advanced. One might suppose that with a weak carcinogen a slight exposure of ionizing radiation or a chemical carcinogen could have a large effect upon the observed biologic response. This would be true with certain dose-response curves. An alternative explanation would be that intensive irradiation of skin (or other tissue) leads to systemic sensitivity to carcinogenic agents. An indication that the latter may be true is provided by experiments of Gorbman (*20*), who found that irradiation of C57BL mice with the head shielded acted as a cocarcinogenic stimulus in the development of pituitary adenomas. While Gorbman believed that irradiation could have acted as a generalized stressor, its action could equally have been a more specific cocarcinogenic one. Experiments have been initiated to explore this possibility.

Strain Sensitivity to Cigarette-Smoke Condensate

Wynder *et al.* (*21*) observed that Swiss mice were more susceptible to cigarette-tar painting than were C57BL mice. Their report is in line with studies of many workers who have observed differences in skin sensitivity to carcinogens among various mouse stocks. The field has been reviewed by Andervont and Edgcomb (*22*) who studied the response of 7 inbred strains of mice to painting with methylcholanthrene. The present data tend to re-emphasize the importance of this factor in experimental design and in interpretation of results. With a weak carcinogen, differences in sensitivity between strains might account for qualitative differences in experimental results.

Significance of These Experiments in Evaluation of Hazards to Human Beings

The data in this investigation support the experimental data of other workers suggesting that cigarette-smoke condensate is a weak mouse-skin

carcinogen. Since the smoking characteristics of the machines that have been employed to produce cigarette tars differ in various respects, it is clear that mouse-skin carcinogenicity of cigarette smoke probably is not dependent upon small variations in puff characteristics. The cocarcinogenic effect of X rays may provide a tool for the detection of such very weak carcinogens.

It has been pointed out many times that animal experimentation cannot be used as a direct measure of the hazard to human beings associated with an environmental agent. This is particularly true in the present series of experiments. The irradiation employed in these studies was not comparable to that of human environment in regard to quality or quantity.

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PLATE 34

FIGURE 1.—Mouse from group V illustrating extent of trauma caused by "sandpapering."

FIGURE 2.—Mouse from group XII illustrating extent of irradiation. The irradiated area is indicated by scarring and lack of pigmentation in the hairs.

FIGURE 3.—Mouse from group X showing a large squamous-cell carcinoma and several papillomas. The approximate irradiated area is indicated by the square.

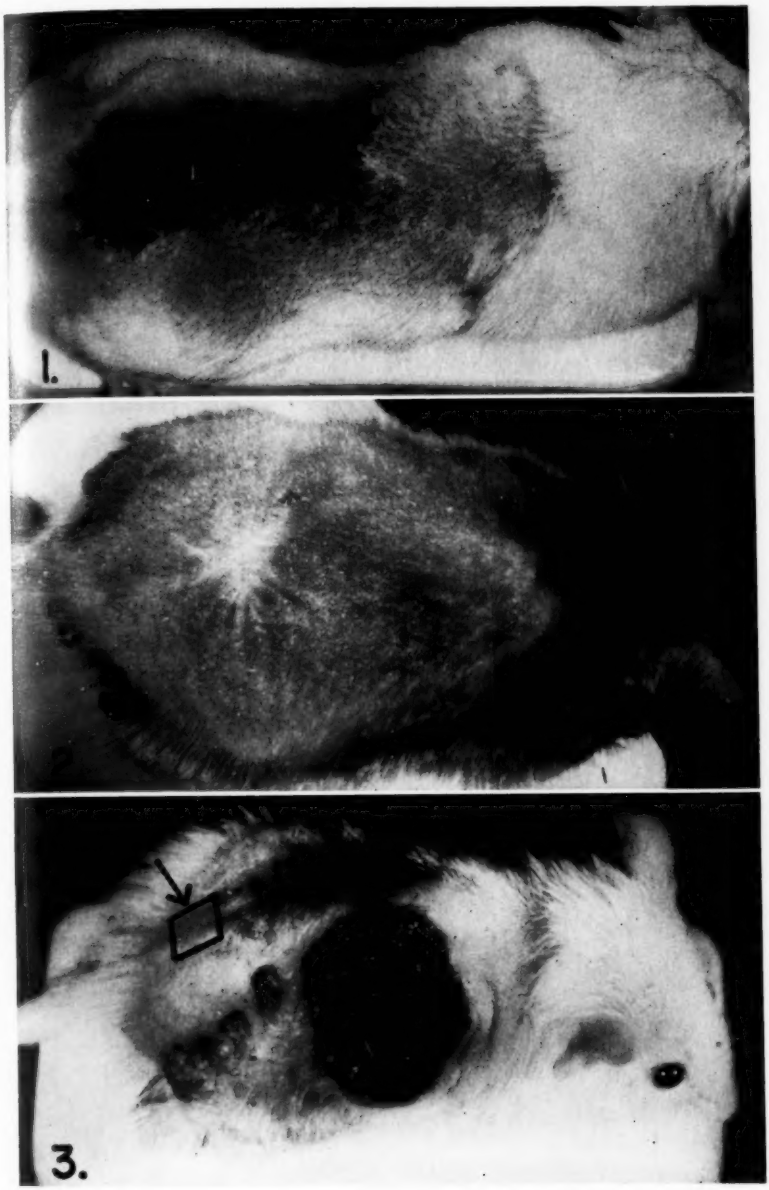

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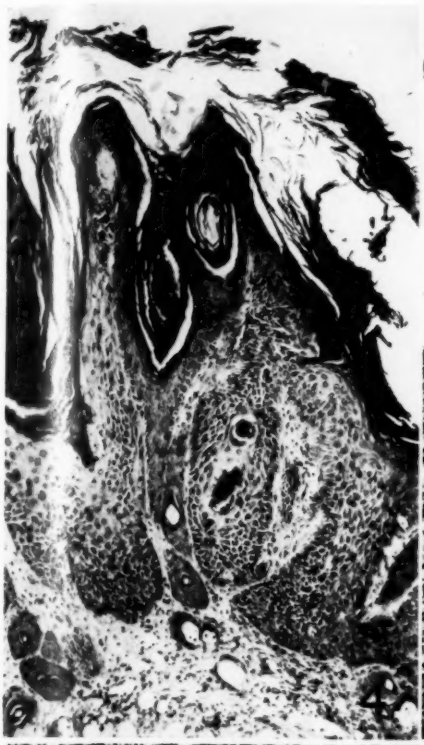
PLATE 35

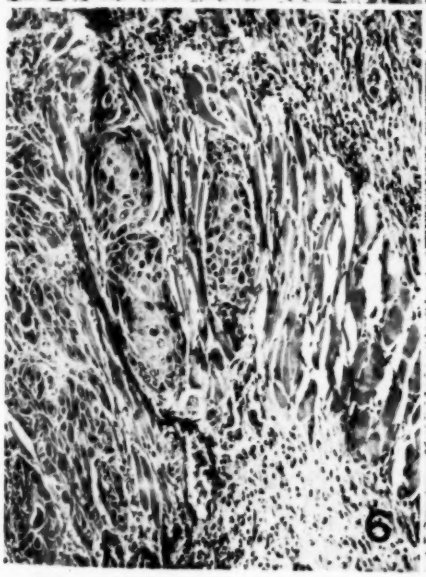
FIGURE 4.—Representative verrucose papilloma. Many of such lesions would probably progress to invasive carcinoma.

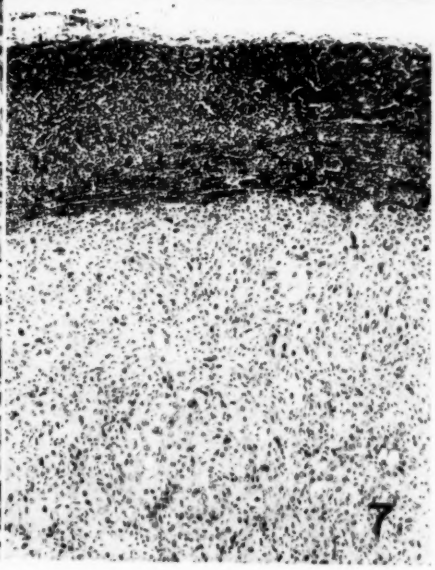
FIGURE 5.—Invasive squamous carcinoma with pearl formation. *Note* verrucose papillary structure similar to that in figure 4.

FIGURE 6.—Invasion of body-wall muscle by squamous carcinoma.

FIGURE 7.—Section of regional lymph-node metastasis. Metastases to the lungs were common, while mestastases to the liver and other organs were rare.







Localization *In Vivo* of Radioiodinated Anti-Rat-Fibrin Antibodies and Radioiodinated Rat Fibrinogen in the Murphy Rat Lymphosarcoma and in Other Transplantable Rat Tumors [1, 2]

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Summary

Radioiodinated rabbit-anti-rat-fibrin antibodies purified by absorption and elution from rat fibrin localized *in vivo*, in the solid form of the Murphy rat lymphosarcoma and in three other transplantable rat tumors, like the anti-ascites-tumor antibodies purified from ascites tumor sediment. Radioiodinated rat and human fibrinogens also localized in a like manner in the lymphosarcoma but not always to the same extent. Several other antiserums, including those prepared against solid and ascites forms of the Murphy-Sturm lymphosarcoma, against ascites forms of three other rat tumors, and against human fibrinogen, contained antibodies with a cross reaction to rat fibrin, which localized in the Murphy lymphosarcoma when purified from rat fibrin. It is suggested that the type of antibody localization described was mediated through fibrinogen during its conversion to fibrin in the tumor mass. The antibody combined with fibrinogen in the circulation and was fixed with it in the tumor.—**J. Nat. Cancer Inst. 22: 413–431, 1959.**

This series of experiments resulted from our attempts to explain a rather singular observation: that antibody, prepared in rabbits against ascites Murphy lymphosarcoma cells and mixed with cleared ascitic fluid, loses its capacity to be absorbed on ascites cells or upon saline-insoluble sediments prepared from them, but nevertheless retains its full capacity to localize *in vivo*, as antibody, in the solid form of the Murphy-Sturm lymphosarcoma.

The inhibitory nature of the ascitic fluid was due to a soluble substance in the fluid which combined with and neutralized the antibody since, as shown later, the extent of inhibition increased with the amount of fluid used. Both ascitic serum and ascitic plasma [4] contained the inhibitor.

[1] Received for publication September 8, 1958.

[2] This work was supported in part by a contract with the U. S. Atomic Energy Commission. Part of this material was presented at a meeting of the American Association for Cancer Research, in Philadelphia, April, 1958.

[3] The authors wish to acknowledge the assistance of Miss Marilyn Sanden, Mr. Louis Kubal, and Miss Mary Jane Danielson, a summer high-school fellow.

[4] Ascitic serum is the fluid that separates from the clotted whole ascites, whereas ascitic plasma is the fluid obtained when clotting is prevented by heparin or other agents, and the cells are removed by centrifugation.

It was subsequently found that rat blood plasma acted similarly and neutralized the ability of antibody to combine with cells *in vivo* but did not neutralize its ability to localize *in vivo* in a solid tumor. Rat serum did not show this property; therefore, it appeared that the neutralizing substance was related to a substance removed from rat blood during the clotting process. If a neutralizing substance were present in rat plasma, however, it would be difficult to see how localization could take place *in vivo* in the presence of a high concentration of inhibitor, since antibody sites would be saturated, unless the combination constant of antibody with tumor antigenic sites were much higher *in vivo* than *in vitro*.

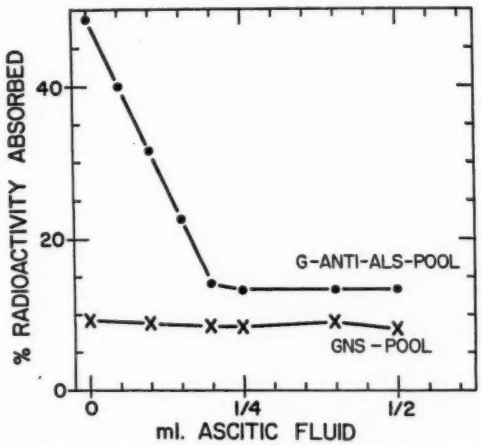
An alternative explanation was that the neutralizing substance was acting as a carrier for the antibody. In this event, the localization properties previously ascribed to antibody would belong to this neutralizing substance. Therefore a study was made of fibrinogen, which is a substance that is present in blood plasma and ascitic serum, but not in blood serum.

Antibodies against rat fibrin, when radiolabeled with I^{131} and concentrated by absorption on, and elution from, rat fibrin, localized specifically and to a high degree in the Murphy lymphosarcoma of rats that had received an intravenous injection of the material. Moreover, radioiodinated fibrinogen itself localized in an apparently like manner. Anti-rat-fibrin antibodies and rat fibrinogen also localized, in three other transplantable rat tumors, like the homologous antitumor antibodies.

Preliminary Experiments

Neutralization of antibody in vitro *by ascitic fluid.*—When ascitic fluid was added to radioiodinated anti-ascites-Murphy-lymphosarcoma antibody (specifically purified by absorption and elution from tumor sediment) the ability of the antibody to be absorbed on sediment decreased markedly (text-fig. 1). The difference here between the antibody and normal globulins may or may not be due to a small amount of antibody which cannot be neutralized by ascitic fluid. This difference was not found for all pools of serum used. For values within a run, the precision of measurement was over 98 percent as shown by triplicate determinations made for each amount of neutralizing substance tested; however, variations occurred between runs made at different times with different preparations of ascitic serum or plasma. In all cases, freshly drawn ascitic fluid was the most potent source of neutralizing substance.

Antibody was purified from the same pool of antiserum globulin as that used for previous studies (*1–3*). Ten mg. of globulin was radioiodinated according to our standard procedure (*1*), purified by double elution (*3*) with a yield of 0.2 percent, and diluted to 100 ml. with borate-buffered (*p*H8) physiological saline. For testing, 2 ml. portions of this solution and 4 ml. of normal rabbit serum were mixed with the ascitic serum and enough buffered saline to bring the final volume to 8 ml., and then added to tubes containing 20 mg. of ascites lymphosarcoma sediment. After 1 hour at 37° C., the sediments were separated from the mixture by



TEXT-FIGURE 1.—Absorption of purified radioiodinated rabbit-anti-ascites-lymphosarcoma-antiserum globulin (G-anti-ALS-pool) and of normal radioiodinated rabbit globulin (GNS-pool) on ascites lymphosarcoma sediment in the presence of varying amounts of ascitic serum from the ascites tumor. *See* text for details.

centrifugation, washed twice by resuspension in 10 ml. volumes of buffered saline and recentrifugation, and then assayed for the presence of absorbed radioactive globulin.

Localization in vivo *of antibody neutralized by cleared ascitic serum.*—An antibody eluate was prepared as before, diluted 1/10 with buffered saline, and divided into 2 parts; 1 part was mixed with enough ascitic fluid to neutralize the antibody and the other was mixed with a similar volume of rat serum. A portion of the eluate mixed with ascitic fluid was tested for absorption on tumor sediment and shown to be absorbed only to the extent of 8 percent (control value), whereas the eluate which was mixed with serum was absorbed to the extent of 48 percent. Two ml. portions of these preparations were injected into lymphosarcoma-bearing rats, which were assayed for the presence of radioactivity in their tissues over and above that expected for normal radioiodinated globulins. As shown in table 1, no difference was found between the localization properties of the 2 preparations.

Experiments

Preparation of rat fibrinogen.—In accordance with the procedure used by Hammarsten (*4*) for preparing fibrinogens of several species, rat fibrinogen was precipitated from plasma by a half saturation with NaCl. The preparations thus obtained were 90 percent clottable.

Two volumes of the aqueous NaCl reagent (277.5 gm. NaCl in 1000 ml. solution at 25 to 28° C.) were added slowly (over 5 minutes) and with thorough but gentle stirring to 1 volume of plasma (usually about 100 ml. containing citrate or heparin). After 1 hour at room temperature the mixture was centrifuged, and the precipitate was redissolved in distilled

TABLE 1.—*Localization of neutralized anti-lymphosarcoma antibody* in vivo *in rats bearing the Murphy lymphosarcoma*

Preparation assayed	Assay *in vitro* (*percentage of purified antibody absorbed on ascites lymphosarcoma sediment*)	Assay *in vivo* (*percentage of injected radioactive dose localized in tissues 1 day after injection, averaged from 4 rats*)				
		Tumor*	Liver	Kidney	Spleen	Blood†
Antibody (purified antibody mixed with normal rat serum)	48. 3	22. 0	2. 7	0. 38	0. 35	22
Neutralized antibody (purified antibody mixed with cleared ascitic serum from ascites lymphosarcoma)	7. 9	21. 6	3. 1	0. 35	0. 35	25

*Includes in each case a background localization of 4.5 percent.
†Blood volume estimated at 10 percent of whole rat weight.

water. The salt precipitation was repeated, and the product either was used for the preparation of fibrin or was dialyzed against 0.9 percent saline and lyophilized. Human fibrinogen was prepared in the same way.

Preparation of rat fibrin.—The fibrinogen from 100 ml. plasma was diluted to 1 liter with buffered saline. To this were added 5 ml. 0.2 M calcium acetate and 1000 NIH units bovine thrombin (Parke, Davis & Company). The fibrin was wound around a glass stirring rod, as it formed, washed with distilled water, and lyophilized. The dried fibrin was then ground to a fine powder and stored at −20° C. until used. Human fibrin was prepared in the same way.

Preparation of antifibrin antiserum.—Each of 4 rabbits was injected intraperitoneally, thrice weekly for 3 weeks, with 20 mg. rat fibrin suspended in 5 ml. saline for each injection, and was exsanguinated the 6th day after the last injection. The serums from all 4 rabbits were pooled and fractionated for globulin by a sodium-sulfate procedure described elsewhere (*1*). Anti-human-fibrinogen antiserum was similarly prepared. The remainder of the antiserums was also prepared according to this protocol.

Purification of anti-fibrin antibodies.—The pooled antiserum globulin was radioiodinated, by a procedure previously described in detail (*1*), in portions of 20 mg. each (final volume, 20 ml.). Each portion was purified according to our double-elution procedure (*3*), with some variation; 100 mg. portions of fibrin powder were homogenized in 10 ml. volumes of normal rabbit serum (Potter-Elvehjem homogenizer) and added to the radioiodinated globulin solution. Homogenization was necessary for successful handling of the fibrin. After absorption for 1 hour at 37° C., and washing, the sediment was suspended in 10 ml. hot buffered saline at 64° C., and gently agitated for 20 minutes. The temperature was higher and the time of elution was longer than that used previously since this seemed to increase the yield. The eluate was added to a second por-

tion of fibrin, similarly suspended in serum, for a second absorption and elution. The final eluate was brought to the desired volume and to 2 percent normal rabbit serum for injection, and assayed in rats 1 day later, as described elsewhere (*1*, *3*).

Radioiodination of rat fibrinogen.—For each experiment in which fibrinogen was studied, 20 mg. of fibrinogen was radioiodinated by the same process used for antiserum globulins (*1*) and with the same amount of iodine. The yield of coupled iodine was 25 percent as in the case of globulin, and there was an average of 2 iodine atoms per molecule of fibrinogen (taking the molecular weight of rat fibrinogen to be 340,000 [5]). The labeling with I^{131} from Oak Ridge and with I^{133} from Brookhaven gave similar results. Since fibrinogen, unlike antibody, did not need to be further purified before assay, considerably less radioactivity was required with the carrier iodide—usually 1 μc. I^{131} per mg., rather than the 200 μc. per mg. usually coupled to whole antiserum globulin before purification. The radiolabeled fibrinogen preparations ranged from 86.7 to 90.1 percent clottability with thrombin as based on radioactivity counts of washed fibrin so produced.

Preparations for the paired label experiments.—In some of the experiments with globulins and/or fibrinogens described here, a paired label technique was used for more precise comparison of the properties of the proteins. One protein would be labeled with I^{131} and another with I^{133} and the 2 would be mixed prior to injection in tumor-bearing rats. The assays for each label in the tissues of the rats were carried out exactly as described previously for the assays of paired labeled globulins (*8*).

Assays in rats for localization of radioactivity.—As in experiments previously described (*1–3*, *8*), each solid-tumor-bearing rat was injected intravenously with 2 ml. of a radioactive preparation and assayed 18 to 22 hours later for radioactivity in its saline-perfused tissues.

Results and Discussion

Localization of antifibrin.—The data in table 2 show that there are antibodies in each of the 4 anti-ascites-tumor antiserums and in the 2 antiserums prepared against solid and solid-ascites forms of the Murphy tumor, which cross-react with rat fibrin *in vitro* and, when purified from rat fibrin, localize substantially in the solid form of the Murphy lymphosarcoma [6] and not in normal tissues. Of the 3 anti-normal tissue antiserums tested, only antilung antiserum showed the presence of such an antibody.[7] The anti-rat-fibrin antiserum, as expected, was also shown to contain antibodies capable of localizing in the tumors.

The data in table 3 show that when the anti-lymphosarcoma antibody was compared to anti-rat fibrin and anti-human-fibrinogen antibodies for its cross-reaction capacity in rat fibrin and human fibrin, the cross

[5] The molecular weight of rat fibrinogen has not been reported, but 340,000 has been reported for bovine (*5*) and human (*6*) fibrinogens. Blombäck and Laurent (*7*), moreover, estimated 350,000 as the molecular weight of bovine, sheep, and goat fibrinogens, but 490,000 for horse fibrinogen.

[6] Preliminary data were reported in reference *9*.

[7] It is difficult to clear the lung of plasma proteins, including fibrinogen, by perfusion.

TABLE 2.—*Localization of antibodies purified from rat fibrin* in vivo *in rats bearing the Murphy lymphosarcoma*

Rabbit antiserum	Percentage of original globulin in purified preparation	Percentage of injected radioactive dose in whole tissues, averaged from 8 rats					
		Murphy lymphosarcoma*		Liver	Kidney	Spleen	Blood†
		Antibody	Background				
Anti-rat kidney	0.03	0	6	3.2	0.5	0.8	21
Anti-rat liver	0.06	1	5	3.1	0.6	0.7	22
Anti-rat lung	0.11	19	6	2.7	0.2	0.4	26
Anti-solid-Murphy lymphosarcoma	0.12	17	6	1.1	0.1	0.2	21
Anti-solid-ascites-Murphy lymphosarcoma	0.14	18	5	1.0	0.1	0.2	25
Anti-ascites-Murphy lymphosarcoma	0.45	26	6	2.2	0.3	0.3	24
Anti-ascites-Miller hepatoma	0.14	20	5	1.3	0.1	0.2	33
Anti-ascites-Walker 256 carcinosarcoma	0.23	19	5	0.7	0.1	0.2	28
Anti-ascites-Novikoff hepatoma	0.11	13	5	1.5	0.1	0.5	29
Anti-rat fibrin	0.56	24	5	1.8	0.1	0.5	33
Anti-rat fibrin	1.06	26	5	1.1	0.1	0.2	27

*Total localization in tumor is the sum of antibody plus background.
†Blood volume estimated as 10 percent of whole rat weight.

reaction was not complete. Three times as much antitumor antibody and anti-rat-fibrin antibody were recovered by the homologous rat fibrin (lines 1 and 2) as by the heterologous human fibrin (lines 4 and 5), and 9 times as much anti-human-fibrinogen antibody (line 6) was recovered by the homologous human fibrin as by the heterologous rat fibrin (line 3). Furthermore, lack of complete cross reaction is apparent in the localization studies *in vivo* since whatever was purified from rat fibrin did localize to the usual 20 percent, whereas whatever was purified from human fibrin localized to a much lesser extent.

These observations confirm those of Hektoen and Welker (*10*) who have reported partial immunological cross reactions in rat and human fibrinogen as well as in another fibrinogens.

Localization of radioiodinated fibrinogen.—The localization of rat fibrinogen *in vivo* was determined simultaneously with the localization of human fibrinogen by the paired radioiodine label technique (*8*). Rat fibrinogen was labeled with I^{131}; human fibrinogen, with I^{133}; and a mixture of the 2 was injected into a series of rats bearing the Murphy tumor. The results are shown in table 4 where it can be seen that the localization properties of the 2 fibrinogens are the same.

Regarding the fibrinogen, we cannot tell what part of the radioactivity in the tumor was due to actual fixation, such as through fibrin formation, and what part was due to an amount of fibrinogen present as a background-producing component of the tissue fluid. Unlike the localizing antibody

TABLE 3.—*Localization of anti-ascites lymphosarcoma, anti-rat fibrin, and anti-human-fibrinogen antibodies purified from rat and human fibrins in vivo in rats bearing the Murphy lymphosarcoma*

Rabbit antiserum	Radioiodinated antibody		Percentage of injected radioactive dose in whole tissues, averaged from 8 rats					
	Purified from	Percentage yield	Murphy lymphosarcoma*		Liver	Kidney	Spleen	Blood†
			Antibody	Background				
1) Anti-ascites-Murphy lymphosarcoma	Rat fibrin	0.45	17	6	1.0	0.08	0.21	24
2) Anti-rat fibrin	Rat fibrin	0.56	24	8	1.0	0.05	0.20	27
3) Anti-human fibrinogen	Rat fibrin	0.11	23	6	1.7	0.12	0.22	27
4) Anti-ascites-Murphy lymphosarcoma	Human fibrin	0.15	9	5	1.0	0.09	0.17	23
5) Anti-rat fibrin	Human fibrin	0.18	5	7	0.4	0.08	0.07	20
6) Anti-human fibrinogen	Human fibrin	0.96	4	4	0.3	0.03	0.05	29

*Total localization in tumor is the sum of antibody plus background.
†Blood volume estimated as 10 percent of whole rat weight.

TABLE 4.—*Localization of I^{131}-rat fibrinogen and I^{133}-human fibrinogen* in vivo *in rats bearing the Murphy lymphosarcoma*

Rat weight (*gm.*)	Tumor weight (*gm.*)	Percentage of injected radioactive dose in whole tissues*					
		Murphy lymphosarcoma		Thyroid		Blood†	
		I^{131}	I^{133}	I^{131}	I^{133}	I^{131}	I^{133}
143	13.0	20.9	20.4	1.7	1.6	16	15
134	8.2	11.5	11.5	1.2	1.2	32	29
128	10.8	18.1	18.0	1.1	1.2	14	14
116	10.0	20.9	20.2	1.3	1.2	16	14
138	13.3	18.1	17.9	1.1	1.1	18	17
131	12.4	23.3	24.2	0.5	0.5	15	15
140	5.9	22.4	24.0	3.3	3.6	27	26
110	8.1	24.9	25.4	0.5	0.5	13	13
133	13.8	16.1	16.7	0.8	0.7	18	18
128	13.0	16.4	17.0	1.2	1.1	23	21
128	8.9	27.0	27.9	1.8	1.9	14	15

*Average localizations of I^{131} and I^{133} in other tissues were, respectively, liver 0.33 and 0.35; kidney, 0.15 and 0.14; spleen, 0.07 and 0.08.

†Blood volume estimated as 10 percent of whole rat weight.

globulin, in which the background components can be estimated from the known action of normal globulin or antibody formed against other substances, there was no compound available here with a chemical make-up similar to that of fibrinogen but divested of any specific localizing activity; however, since fibrinogen of heterologous origin distributes the same as rat fibrinogen, this indicates that when localization of fibrinogen occurs, it most probably comes about by some property specific to fibrinogen which is different from that of γ-globulin, *i.e.*, conversion to fibrin.

The variable manner in which individual rats with Murphy tumors may react to a particular preparation of iodinated fibrinogen can be seen in table 5. There is an additional fall in blood level of radioiodinated fibrinogen in some of the rats and, with this drop, there is a parallel drop in tumor localization and a concomitant increase in thyroid localization, indicating rapid metabolism of the iodinated fibrinogen by the tumor-bearing rat during this active growth stage. These variations have not been observed with iodinated γ-globulin or antitumor antibody. Impli-

TABLE 5.—*Localization of I^{131}-rat fibrinogen (arranged in increasing order of thyroid localization)* in vivo *in rats bearing the Murphy rat lymphosarcoma*

Rat weight (*gm.*)	Tumor weight (*gm.*)	Percentage of injected radioactive dose of I^{131}-rat fibrinogen in whole tissues			
		Thyroid	Tumor	Blood*	Whole rat
151	6.4	2.4	17.2	26.9	70.5
83	5.1	3.2	13.8	24.1	69.6
130	4.8	3.5	9.7	20.9	63.7
157	5.3	8.4	6.3	15.2	56.5
138	2.9	10.7	5.3	14.7	53.4

*Blood volume estimated as 10 percent of whole rat weight.

cation of plasmin with this phenomenon is hard to avoid since fibrinogen and fibrin, but not γ-globulin, appears to be involved.

In order to determine the relative extent of localization of antifibrin antibodies and rat fibrinogen as based on a percentage of the injected dose, we carried out the experiments shown in table 6. Purified anti-rat-fibrin antibodies, labeled with I^{131}, were paired with I^{133}-rat fibrinogen and injected into tumor-bearing rats. The amount of radioactivity found in the tumors 1 day later was different for the 2 proteins, but the blood levels were also quite different. In a similar experiment comparing unpurified I^{131}-anti-rat-fibrin antiserum globulin with I^{133}-fibrinogen (table 7), there was a similar lowered blood level of I^{133}-fibrinogen.

The lower blood values obtained with fibrinogen probably reflect more rapid metabolism as evidenced by the greater thyroid levels. Since more metabolism would decrease the amount of fibrinogen available for localization, we have normalized the tumor-fixed fibrinogen values by multiplying by the ratio of $\frac{I^{131}}{I^{133}}$ in the blood in an attempt to correct for this metabolism. The values thus calculated for fibrinogen localization are quite similar to the values for antibody localization [corrected for background γ-globulin localized (*1*, *2*)]. Data in table 7B are similar to those of table 6B.

The parallel relation between antifibrin localization and normalized fibrinogen localization is additional evidence to support the hypothesis that fibrinogen mediates the localization of antifibrin antibodies.

Localization of anti-rat fibrin and rat fibrinogen in other transplantable rat tumors.—These tumors and the localization of antitumor antibodies in them will be described elsewhere (*9*, *11*). The localization of control globulins (rabbit anti-bovine-γ-globulin and rabbit normal globulin) is shown in text-figure 2. As in the case of the Murphy lymphosarcoma, shown here for comparison, the Walker carcinosarcoma and the Miller and Novikoff hepatomas exhibit high uptakes of injected radioiodinated rabbit globulin, but in a predictable fashion. This is shown by the least-square lines in the figure, which relate the fraction of total dose in the tumor to the fraction of total rat weight attributable to the tumor. The equations for the lines were used to calculate the amount of injected antibody globulin, nonspecifically localized only as background, with which, by subtraction from the total dose, the amount of globulin specifically localized as antibody was estimated.

The localizations of antifibrin and fibrinogen in the tumors (table 8) were determined by using the paired label technique in the same way as that discussed for the Murphy tumor. The normal tissue localizations were no different than usual and are not included here. As in table 6, the tumor fibrinogen value was multiplied by the ratio of the isotopes in the blood, for comparison of fibrinogen localizations with antibody values. The tumor localizations of antifibrin and of fibrinogen were the same in the Murphy lymphosarcoma and in the Miller hepatoma. It can be seen that in the case of the Walker and Novikoff tumors, which

TABLE 6.—*Localization of purified I^{131}-anti-rat fibrin and I^{125}-rat fibrinogen* in vivo *in rats bearing the Murphy lymphosarcoma*

A

Rat weight	Tumor weight	Percentage of injected I^{131}-anti-rat fibrin				Percentage of injected I^{125}-rat fibrinogen			
		Tumor	Liver	Thyroid	Blood*	Tumor	Liver	Thyroid	Blood*
120	8.7	34.3	1.5	0.4	30.4	11.5	0.23	2.2	12.8
121	4.9	28.9	1.3	0.2	38.7	10.2	0.29	1.2	15.5
112	8.6	29.2	1.7	1.0	31.4	8.8	0.31	4.0	12.4
102	11.2	31.3	3.0	1.1	30.2	9.5	0.97	5.8	13.3
114	11.0	23.8	1.6	0.7	31.1	7.2	0.19	7.0	13.0
132	14.6	24.2	1.5	0.7	30.7	6.6	0.25	3.3	11.9

B

Background localization of I^{131}-globulin in tumor (*calculated*)	Antibody localization of I^{131}-globulin in tumor (*total minus calculated background*)	Ratio in blood of I^{131}/I^{125}	Normalized dose of fibrinogen in tumor (*I^{125} in tumor × ratio in blood of I^{131} to I^{125}*)
6.5	27.8	2.38	27.4
3.8	25.1	2.50	25.5
6.9	22.3	2.53	22.2
9.8	21.5	2.26	21.4
8.6	15.2	2.39	17.2
9.8	14.4	2.58	17.0

*Blood volume estimated as 10 percent of whole rat weight.

TABLE 7.—*Localization of unpurified I^{131}-anti-rat fibrin and I^{133}-rat fibrinogen* in vivo *in rats bearing the Murphy lymphosarcoma*

A

Rat weight	Tumor weight	Percentage of injected unpurified I^{131}-anti-rat fibrin				Percentage of injected I^{133}-rat fibrinogen			
		Tumor	Liver	Thyroid	Blood*	Tumor	Liver	Thyroid	Blood*
130	14. 89	11. 1	—	0. 7	26. 0	8. 1	—	3. 1	7. 4
117	12. 42	9. 5	—	1. 0	17. 7	12. 2	—	2. 8	9. 1
131	9. 17	6. 3	—	0. 8	22. 6	11. 3	—	2. 9	11. 8
111	14. 62	11. 2	1. 25	0. 8	27. 4	6. 8	0. 25	2. 3	7. 6
128	7. 78	5. 8	—	0. 9	34. 0	4. 9	—	2. 7	11. 2
107	12. 33	9. 1	—	0. 2	39. 8	3. 9	—	0. 4	15. 9

B

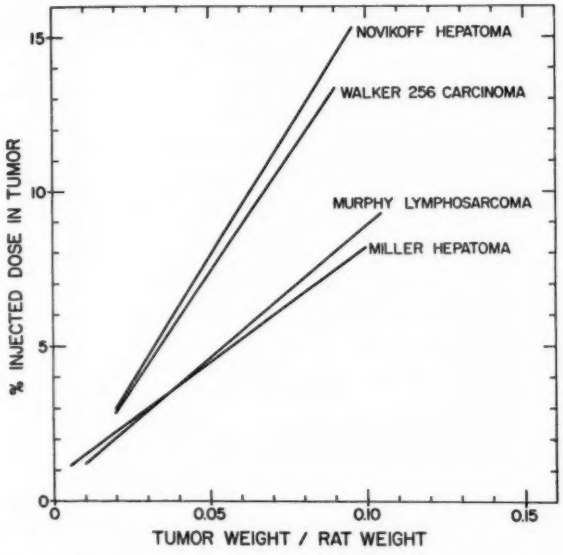
Background localization of I^{131} globulin in tumor (*calculated*)	Deviation of observed values of unpurified I^{131}-anti-rat fibrin in tumors from calculated background values (*total minus calculated background*)	Ratio in blood of I^{131}/I^{133}	Normalized dose of fibrinogen in tumor (*I^{133} in tumor × ratio in blood of I^{131} to I^{133}*)
10. 1	1. 0	3. 50	28. 4
9. 4	0. 1	1. 95	23. 8
6. 3	0. 0	1. 91	21. 6
11. 3	−0. 1	3. 61	18. 8
5. 5	0. 3	3. 04	14. 9
10. 2	−1. 1	2. 50	9. 7

*Blood volume estimated as 10 percent of whole rat weight.

TABLE 8.—*Localization of purified I^{131}-anti-rat fibrin and I^{133}-rat fibrinogen* in vivo *in rats bearing various transplantable rat tumors (8 rats per group)*

Type tumor assayed	Percentage injected dose				Antibody localization in tumor (*total minus calculated background*)	Fibrinogen localization in tumor (*normalized*)
	I^{131}-anti-rat fibrin		I^{133}-rat fibrinogen			
	Tumor	Blood*	Tumor	Blood*		
Murphy lymphosarcoma	29	21	14	13	21	22
Miller hepatoma (line 2)	10	32	5	20	8	8
Walker carcinosarcoma	17	35	10	21	8	17
Novikoff hepatoma	18	42	11	21	9	22

*Blood volumes estimated as 10 percent of whole rat weight.



TEXT-FIGURE 2.—Accumulation 1 day after intravenous injection of radioiodinated nonlocalizing rabbit serum globulin in 4 solid rat tumors as a function of tumor and body size. The lines are plots of least-square equations obtained as described in (*1*). Slopes and ordinate-intercepts of the lines are as follows: Miller, 73 and 0.8; Murphy, 86 and 0.3; Walker, 143 and 0.1; Novikoff, 163 and −0.3. Confidence limits are not shown but are similar to those described for the Murphy tumor (*1*).

exhibit much higher uptakes of normal globulin than the Murphy and Miller tumors, the fibrinogen values are more like the total globulin (antibody plus background) values than the net antibody values.

Conclusions

In retrospect, perhaps it would be more in order to discuss first what we have not shown by these experiments than what we have shown: (*a*) We have not shown that our anti-ascites-lymphosarcoma antibodies are in reality anti-rat-fibrin antibodies—only that antibodies against some strongly antigenic substance within them cross-react to varying degrees with rat fibrinogen and fibrin; (*b*) we have not shown whether or not the neutralizing substance in ascitic fluid is exclusively rat fibrinogen or whether it comes from the host or the tumor cells; (*c*) we have not shown that localization of fibrincgen and anti-fibrinogen in transplanted tumors is any more than an interesting phenomenon associated with certain transplanted tumors.

On the other hand, we have shown that fibrinogen and antifibrin have localization properties, in several transplanted tumors, similar to each other and analogous to those localization properties of antitumor antibodies so far prepared. We have also elucidated the interesting possibility of fibrinogen conversion to fibrin while bound to antibody.

Although we now know a large part of the tumor localization that we had previously observed can be attributed to cross reaction of the antibody with fibrinogen rather than with some specific tumor substance, we are unable to tell what portion, if any, of the tumor localizations observed in the past is due to the localization of specific antitumor antibody, and investigations are being continued in a search for such tumor-specific substances important for fixation of antibodies *in vivo*. Evidence *in vitro* of specific substances (other than fibrinogen-like substances) on tumor cell surfaces is available since we have found that fibrinogen does not inhibit completely the pickup of antitumor antibody on ascites cells of the Miller hepatoma. Moreover, the work of Wissler and Flax (*12*) shows that Ehrlich cells pick up anti-Ehrlich ascites tumor antibodies in the presence of ascitic fluid.

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Application of Millipore Filtration Technique to Cytologic Material [1]

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Summary

A millipore filtration technique for preparing fluid specimens for cytologic examination is described. This method was designed for simplicity and for recovery of the maximum number of cells from all specimens, regardless of volume or cell concentration. The morphology of the cells was well preserved.—J. Nat. Cancer Inst. 22: 427–431, 1959.

There are several drawbacks in previous methods of centrifuging small quantities of fluids and preparing secretions for cytologic examination. Frequently, the few cells in some of the fluids are lost during transfer of the sediment from the centrifuge tube to the slides or the cells fail to stick to the albumin-coated slides and are washed off during the staining procedure. Furthermore, in processing fluids containing blood, the fact that the erythrocytes often obscure the epithelial cells makes evaluation of the material difficult.

A method to concentrate cancer cells suspended in large quantities of fluid by the use of millipore filters has been described by Seal (*1*). In an attempt to eliminate the drawbacks of the centrifugation technique, an adaptation of the millipore method to a variety of fluids containing few cells has been under study in our laboratory for the past year. A description of the millipore filtration technique and the results are presented here.

Materials and Methods

The specimen is diluted by adding 25 ml. of 0.85 percent saline per ml. of specimen. The diluted specimen is filtered through a 47-mm. SM Millipore filter [3] with 20-mm. mercury negative pressure. The volume of material put through one filter is varied inversely with the anticipated cell content. Near the end of the filtration process the specimen on the filter is washed with 0.85 percent saline and followed by the addition of

[1] Received for publication October 7, 1958.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[3] Millipore Filter Corp., P. O. Box 297, Bedford, Watertown, Mass.

10 ml. of modified Carnoy's fixative (*2*). It is important that fluid remain on the specimen throughout the filtration process, for air drawn into the system tends to distort the cell morphology. After filtration, the filter is placed in modified Carnoy's fixative in a polyethylene millipore filter holder designed in our laboratory (fig. 1).[4] It is stored in modified Carnoy's fixative before staining by the standard Papanicolaou technique (*3*). After dehydration with alcohol and clearing in xylene, the filter is removed from the holder and mounted cell side up on a 3 × 2 inch glass slide upon which approximately 6 drops of Permount have been placed. Another 5 or 6 drops of Permount are placed on the filter which is then covered with a 49- × 50-mm. #1 thickness coverglass (fig. 2).

Results and Discussion

The technique described has been successfully applied to a variety of specimens including urine, pleural fluid, ascitic fluid, gastric fluid, bronchial washings, cyst fluid, cerebrospinal fluid, and synovial fluid. Representative cells from various fluids are illustrated in figures 3 to 10. The method is equally adaptable to large and small amounts of fluid, and it assures the maximum recovery of cells, regardless of the original cell concentration. The cellular material which collects on the filter adheres to it firmly, and thus during the staining procedure the possibility of cell loss is reduced. Suspension of the cells in fluid media during the process of filtration results in an even distribution of the material on the final preparation in contrast to the smeared preparations of the conventional type.

Cellular detail is well preserved by the millipore filtration technique. This method is more rapid, since it eliminates the need for centrifugation.

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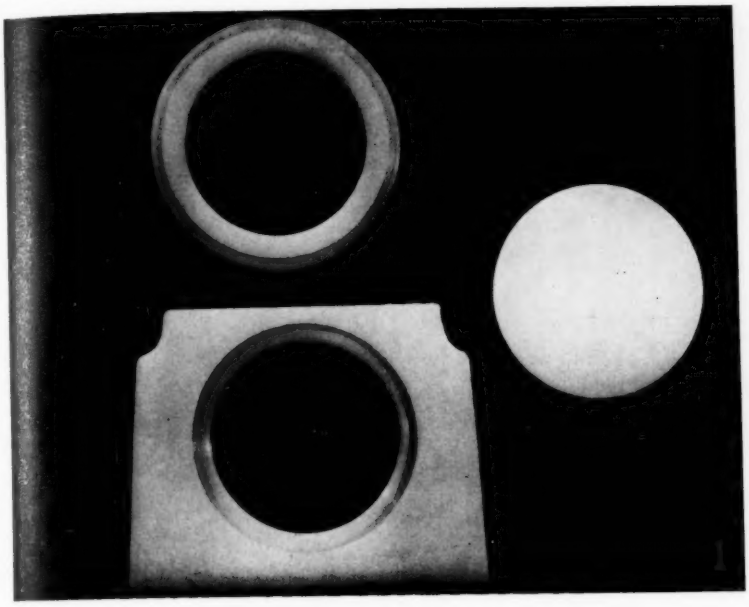
[4] The millipore filter holder was designed with the assistance of Mr. Philip Joram, Plastics Unit, Medical Arts Section, Scientific Reports Branch, Division of Research Services, National Institutes of Health, Bethesda, Md., and manufactured by Duke Bontz & Co., 8409 2d Avenue, Silver Spring, Md.

PLATE 36

FIGURE 1.—Millipore filter holder. Filter fits into recessed area in holder and is held in place by ring. Filter and holder are then carried in a staining tray through the staining solutions.

FIGURE 2.—Stained filter mounted on glass slide ready for examination.



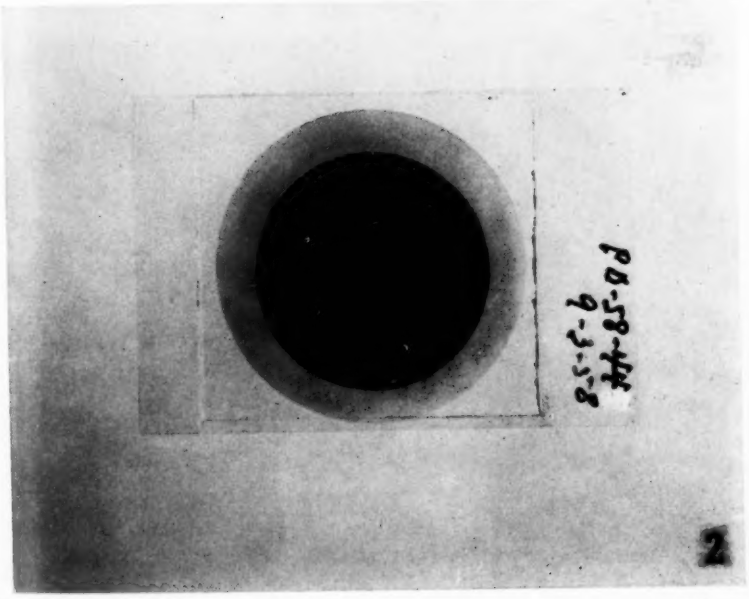


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PLATE 37

Representative cells on millipore filter preparation from various fluid specimens.

FIGURE 3.—Adenocarcinoma cells in a cluster with scattered mesothelial cells, lymphocytes, and polymorphonuclear leukocytes from ascitic fluid. Patient had an adenocarcinoma of the pancreas. × 480

FIGURE 4.—Adenocarcinoma cells and polymorphonuclear leukocytes in pleural fluid. Same patient as figure 3. × 970

FIGURE 5.—Neoplastic cells and leukocytes in urine. Patient had a bladder carcinoma. × 440

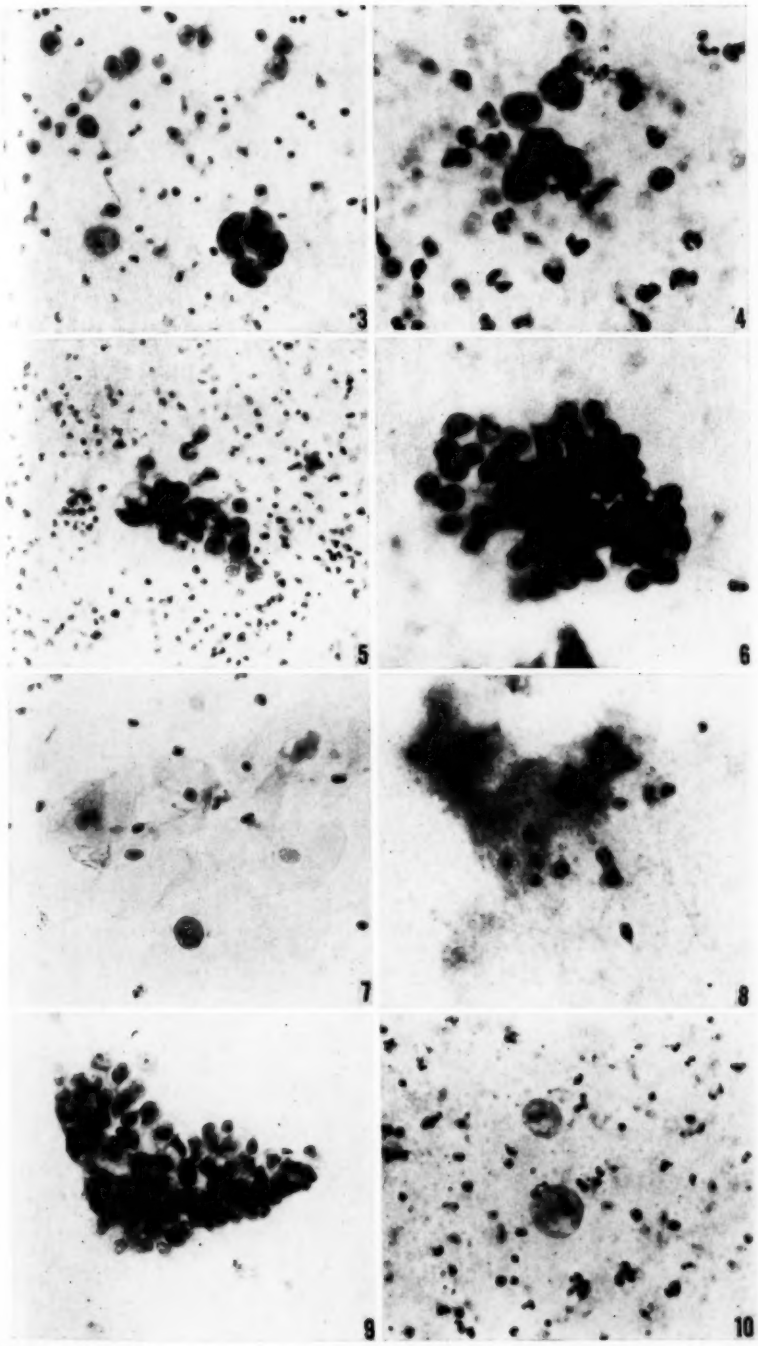
FIGURE 6.—Columnar epithelial cells from gastric washings. × 900

FIGURE 7.—Squamous epithelial cells from vaginal fluid. × 440

FIGURE 8.—Ependymal cells from cerebrospinal fluid. × 440

FIGURE 9.—Duct cells from breast secretion. × 440

FIGURE 10.—Lipide-laden macrophages from synovial fluid. × 480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A Clinical Study of the Comparative Effect of Nitrogen Mustard and DON in Patients With Bronchogenic Carcinoma, Hodgkin's Disease, Lymphosarcoma, and Melanoma [1,2]

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Summary

In a cooperative study involving 11 Veterans Administration Hospitals, the effect of HN_2 (nitrogen mustard) and DON (6-diazo-5-oxo-L-norleucine), an "antibiotic" isolated from *Streptomyces*, was compared in patients with bronchogenic carcinoma, Hodgkin's disease, lymphosarcoma, and melanoma. HN_2 was given intravenously in a dose of 0.2 mg. per kg. of body weight for 2 consecutive days. DON was given in a single oral dose of 0.2 mg. per kg. daily for 30 days. The effects were compared by measuring changes in tumor size, by the performance status of the patient, and by the impression of each investigator on the over-all effect. Neither drug was effective in malignant melanoma. HN_2 was superior to DON in Hodgkin's disease and lymphosarcoma and slightly better than DON in bronchogenic carcinoma.—**J. Nat. Cancer Inst. 22: 433–439, 1959.**

[1] Received for publication July 15, 1958.

[2] This study was conducted through the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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In the evaluation of chemotherapeutic agents for the treatment of cancer, the experience of any single investigator is usually too limited for an adequate statistical analysis. A chemotherapy study program was organized so that large numbers of patients with certain tumors, hospitalized in 11 cooperating Veterans Administration Hospitals, could be treated under substantially identical conditions and could be considered as a single group. This would afford an opportunity to screen accurately and quickly the newer drugs becoming available.

The first study conducted by this group was designed to compare the efficacy of nitrogen mustard [3] and DON (6-diazo-5-oxo-L-norleucine), an "antibiotic" isolated from *Streptomyces*, in the treatment of bronchogenic carcinoma, Hodgkin's disease, lymphosarcoma, and melanoma.

The use of the alkylating agents in the treatment of these tumors has been studied for at least the past 10 years (*1*). Nitrogen mustard and some of its congeners have been found to exert a beneficial influence in some patients with Hodgkin's disease and lymphosarcoma and to have little or no effect in bronchogenic carcinoma and malignant melanoma (*2*).

DON has been shown, in studies both *in vivo* and *in vitro*, to depress significantly the incorporation of orotic and ureidosuccinic acids into nucleic acid cytosine, probably by interfering with one of the reactions leading from a metabolite containing the uracil moiety to one containing cytosine (*3*). This antimetabolite has demonstrated inhibitory effects on mouse sarcoma and other animal tumors (*4–7*).

A clinical trial of DON was begun in June, 1955, by Magill and his colleagues (*8*). The dosage ranged from 0.1 to 0.3 mg. per kg. per day, either orally or intramuscularly. There appeared to be a definite regression of the tumor in 7 of 47 patients with widespread carcinomatosis of varied types. The effect was not rapidly apparent and appeared only after a few weeks of therapy. Reversal of hypercalcemia was noted in 4 additional patients. It was suggested that DON might have a "carcinostatic" rather than "carcinolytic" effect. Eighty-three percent of the patients experienced some gastrointestinal toxicity within 7 to 14 days and another 24 percent developed leukopenia or thrombopenia or both (*9*).

All patients who had one of the tumors included in the study, and who were admitted to the cooperating hospitals between January 1, 1957 and August 30, 1957, were considered for the clinical study, provided they had primary or metastatic lesions that could be measured, except in patients with bronchogenic carcinoma in whom the primary pulmonary lesion had to be measurable whether or not secondary lesions were available. Patients were not accepted if their disease was amenable to classical therapy for cure or extended palliation (*e.g.*, resection in bronchogenic carcinoma or irradiation in patients with Hodgkin's disease), or if they were deemed moribund on admission.

Patients who had received either roentgen-ray therapy or alkylating agents were not admitted to the study until 8 weeks after the therapy

[3] *Chemical Abstracts'* nomenclature: 2,2'-dichloro-N-methyldiethylamine hydrochloride.

had been completed and there was evidence of either a failure of response or a relapse. The lesions selected for measurement in patients with Hodgkin's disease or lymphosarcoma had not been irradiated for at least 3 months. Patients with melanoma were not accepted if they had ever had alkylating agents. Patients with pancytopenia due to drug-induced hypoplasia of the bone marrow were excluded. This was defined as a peripheral white blood-cell count of below 3,000 cells per $mm.^3$, marked reduction of platelet count, and hypocellular marrow. If steroids had been given previously, their use was discontinued for at least 2 weeks before treatment.

Only patients with a proved histological diagnosis were accepted. All biopsy material from patients with lymphomas was reviewed by the same pathologist—Dr. Arthur Allen, Professor of Pathology, University of Miami—and classified as either Hodgkin's disease or lymphosarcoma. Reticulum-cell sarcoma was included with lymphosarcoma.

Because of the strict criteria, only 113 patients were acceptable for the study out of the 804 patients, with these diseases, who were admitted to the cooperating hospitals (table 1).

Each hospital was provided with a series of sealed envelopes prescribing the drug to be used. These envelopes were randomized by the biostatistician.

TABLE 1.—*Total number of patients acceptable*

Disease	Accepted	Rejected	Total patients screened
Bronchogenic carcinoma	52	440	492
Hodgkin's disease and lymphosarcoma	51	218	269
Melanoma	10	33	43
Total	113	691	804

Patients were observed for a sufficient period to document tumor size and to determine their clinical status, which was given a numerical rating of 10 to 100 percent. Those with a rating of 80 to 100 percent were able to carry on normal activity, with few or no symptoms of disease. Those with ratings of 50 to 70 percent were unable to work but could take care of themselves. Those with lesser performance ratings were ill enough to require hospitalization.

All the lesions were measured by calipers at least twice within a week prior to therapy and twice a week afterwards. The size of the tumors was assessed without reference to the previous measurements. Whenever possible, two dimensions were recorded for each lesion, but in some of the pulmonary masses, only one diameter could be measured. The reliability of the measurement technique is analyzed in table 2. Not more than 11 percent of the two sets of pretreatment measurements showed a decrease of more than 20 percent.

TABLE 2.—*Pretreatment variation in tumor size*

Disease	Number of patients	Total No. of lesions measured	Percent disappeared	Percent decreased by 20% or more	Percent increased or stable
Hodgkin's disease	32	78	0	10	90
Lymphosarcoma	19	62	0	5	95
Bronchogenic carcinoma	52	85	0	5	95
Melanoma	10	28	0	11	89

Nitrogen mustard was given intravenously in a dose of 0.2 mg. per kg. body weight for 2 consecutive days. DON [4] was given in a single oral dose of 0.2 mg. per kg. daily for a period of 30 days. Treatment was temporarily suspended if severe toxicity developed and was reinstituted when the signs of toxicity abated.

The effect of the drugs on the patients was assessed at the end of 30 days, excluding those few who received nitrogen mustard and died, and those who were removed from the study before 30 days. Of the patients who received DON, none was considered in the final tabulation, unless the patient had received the drug for 30 days.

Results

The results were compared in three ways. A decrease of 20 percent or more in the size of the measurable lesions was considered to represent a detectable change in the tumor. Changes in performance status were the second parameter of comparison; a rise of 20 percent or more was held to be an improvement. Each of the principal investigators voted on his impression of the over-all effect on each patient. These votes were tabulated and classified in two groups: good and poor. The majority vote determined in which group the response was to be classified.

The comparative results of these two drugs on tumor size are listed in table 3. In patients with Hodgkin's disease, who were treated with nitrogen mustard, 80 percent of the measured lesions decreased in size or dis-

TABLE 3.—*Comparison of effect of HN_2 and DON on tumor size*

Disease	Drug	Number of lesions measured	Percent disappeared	Percent decreased by 20% or more	Percent increased or stable
Hodgkin's disease	HN_2	77	35	45	20
	DON	41	7	40	53
Lymphosarcoma	HN_2	24	17	30	53
	DON	26	0	10	90
Bronchogenic carcinoma	HN_2	56	0	26	74
	DON	24	0	4	96
Melanoma	HN_2	14	0	0	100
	DON	13	0	0	100

[4] DON supplied through the courtesy of Parke, Davis & Company.

appeared as compared to 47 percent in the DON group. The difference in these results suggests that nitrogen mustard is superior to DON as to influence on tumor size. Since no untreated control group was used, the actual effect over and above the possible spontaneous changes known to occur in this disease cannot be evaluated, but it would appear that DON exerted some beneficial effect.

However, in patients with lymphosarcoma, who were treated with DON, only 10 percent of the measured tumors decreased. Since this is in the range of measurement variability, it is probable that DON was ineffective in this disease. Nitrogen mustard produced a decrease or disappearance of 47 percent of the measured tumors.

Similar results were obtained in the patients with bronchogenic carcinoma—26 percent of the lesions decreased in the group treated with nitrogen mustard as compared to 4 percent in the group treated with DON. Neither drug was effective in melanoma. Similar differences, although not so striking, between the responses to the two drugs can be demonstrated by comparing the changes in performance status (table 4) and the over-all evaluation (table 5).

TABLE 4.—*Comparison of effect of HN_2 and DON on performance status response*

Disease	Drug	Total No. of patients	Improvement of 20 points or more (*percent*)	No change (*percent*)
Hodgkin's disease.....	HN_2	21	45	55
	DON	11	20	80
Lymphosarcoma.....	HN_2	12	10	90
	DON	7	0	100
Bronchogenic carcinoma.	HN_2	34	10	90
	DON	18	0	100
Melanoma.....	HN_2	5	0	100
	DON	5	0	100

The dose of DON employed produced toxic reactions in 80 percent of the patients. In 38 percent the drug had to be discontinued temporarily because of untoward reactions, but then it could be given again. Toxicity appeared in about 14 days. The types of toxic reactions are listed in table 6. Forty-four percent of the patients receiving nitrogen mustard had some toxic reactions. Nausea and vomiting associated with the administration of the drug was common, and leukopenia and thrombopenia appeared in 2 or 3 weeks but did not persist (table 7). No effect in the peripheral blood count was noted with DON over this 30-day period.

From the toxicity results it appears that the maximum tolerated dose of DON was used. The 30-day period of trial was also adequate. By that time there had been an increase in the size of the tumor in almost all patients, so that we were unable to demonstrate any "carcinostatic" effect. In those who had a decrease in tumor size, the changes were

TABLE 5.—*Comparison of effect of HN_2 and DON as measured by votes of 11 principal investigators based on over-all effect*

Disease	Drug	Total No. of patients	Good (*percent*)	Poor (*percent*)	
Hodgkin's disease	HN_2	21	80	20	$X^2 = 18.6$*
	DON	11	10	90	
Lymphosarcoma	HN_2	12	17	83	$X^2 = 0.4$†
	DON	7	0	100	
Bronchogenic carcinoma	HN_2	34	12	88	
	DON	18	0	100	
Melanoma	HN_2	5	0	100	$X^2 = 0.9$†
	DON	5	0	100	

*Difference statistically significant ($P<0.1\%$).
†Differences not statistically significant.

TABLE 6.—*DON toxicity (41 patients)*

Type of toxicity	Number of patients
Stomatitis	15
Vomiting	10
Anal soreness	7
Diarrhea	7
Anorexia	4
Metallic taste	2
Leukopenia (under 3,000/mm.^{3} WBC)	0
Thrombocytopenia	0

TABLE 7.—*HN_2 toxicity** (*72 patients*)

Type of toxicity	Number of patients
Leukopenia (under 3,000/ml.^{3} WBC)	14
Thrombocytopenia	6

*Almost all patients receiving HN_2 experienced some vomiting, but this was considered an expected side effect rather than a toxic reaction.

temporary, and the tumor started to grow shortly after the 30-day period while the patient was still receiving DON.

This study has demonstrated the superiority of nitrogen mustard to DON, under the experimental conditions of the study, in Hodgkin's disease and lymphosarcoma. In patients with bronchogenic carcinoma, neither drug was very effective but nitrogen mustard did exert some influence. In malignant melanoma neither drug produced any beneficial effect.

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Announcements

Cancer Chemotherapy Reports

The Cancer Chemotherapy National Service Center, Public Health Service, U. S. Department of Health, Education, and Welfare, is now issuing a series of technical reports called *Cancer Chemotherapy Reports* covering all phases of cancer chemotherapy research. Material to be included will be in the form of progress reports of current research, communications, screening data, announcements, bibliographies, abstracts, and reports from interested investigators, the Cancer Chemotherapy National Service Center staff, and its technical panels.

Cancer Chemotherapy Reports may be obtained free of charge from:

Publications Officer
Cancer Chemotherapy National Service Center
National Cancer Institute
National Institutes of Health
Bethesda 14, Maryland

International Congress of Gastroenterology

The 6th Meeting of the Association of the National European and Mediterranean Societies of Gastroenterology, organized by the Association of Dutch Gastroenterologists, will be held in Leiden, the Netherlands, from April 20 to 24, 1960. The main themes of the conference will be: 1) Pathology of the small intestine; 2) hepatitis, *cirrhosis hepatis*, and their possible connection. Panel discussions, lectures, and films will be arranged in connection with these themes and related subjects. Scientific and technical-commercial exhibits will be shown.

Original reports on these subjects or any other gastroenterological subject, either clinical or in the field of the basic sciences, may be submitted and will be accepted for panel discussion or publication if approved by the Scientific Program Committee. Titles for papers together with a summary of not more than 200 words should be sent not later than August 1, 1959, to Dr. B. K. Boom, Congress Office, Department of Gastroenterology, University Hospital, Leiden, the Netherlands. Official language: English; working languages: English, French, German, Spanish (with simultaneous translation).

Registration fee (including an official reception, a banquet, and an excursion) for full member: U. S. $45.; for accompanying family members: U. S. $20. After November 1, 1959, the registration fee for full members will be U. S. $55. The fees quoted in U. S. dollars can be paid also in Dutch guilders or any other transferable currency.

For further information and for registration apply to the Congress Office of the 6th Meeting of the Association of the National European and Mediterranean Societies of Gastroenterology, Department of Gastroenterology, University Hospital, Leiden, the Netherlands, or to the Secretary of the national societies of gastroenterologists.

T. C. A. Summer Course in the Principles and Techniques of Tissue Culture—1959

The Tissue Culture Association is again sponsoring a Course of instruction in the principles and techniques of cell and tissue culture. The Course will be under the direction of Dr. John Paul, University of Glasgow, Scotland, and will be given at the University of Colorado School of Medicine, Denver, Colorado, from July 6 through July 31. Tuition: $100.

This is an intensive 4-week Course dealing with the structure and function of living cells, techniques of tissue cul-

ture, and interpretation of results. It is designed to give to responsible investigators a background of general information on cultured cells and an opening wedge of training in the application of the method to problems in several current areas of research.

Because of limitations in laboratory space, only 15 active participants will be accepted. These places will be allocated to those with no previous tissue-culture experience who, in the committee's opinion, will derive benefit from the Course. In addition, 15 auditors will be accepted. Places as auditors will be allocated to senior applicants with predominantly administrative responsibilities and to those who have already had practical experience of tissue-culture techniques.

The morning work includes lectures and discussions on practical aspects of tissue-culture techniques and demonstrations of procedures to be used in the laboratory. Each active participant prepares and manages his own cell cultures. Auditors attend all sessions but do not carry out the laboratory exercises. Afternoons afford opportunity for library work and for consultation with the staff concerning the projects contemplated by each of the class members. Evening lectures, Monday through Friday, by members of the staff and by distinguished guest lecturers cover various fields of research in which the tissue-culture method has been used to advantage. The evening lectures are open to members of the Faculty of the University of Colorado and to wives and families of participants in the Course.

The Course is designed specifically for postgraduates (M.D. or Ph.D.) who plan to use cultured tissues in their research or teaching. Requests for application forms should be addressed to Dr. Mary S. Parshley, College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York, and should be completed and returned to her not later than April 1. Successful candidates will be notified about April 15.